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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

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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.

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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

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enteric pathogenicity, BCoV strains 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: TGTAACGACGGCCAGT; 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/](http://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. Pair-wise 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

Table 1  
Nucleotide differences between AH65-E and AH65-R and mutations in AH65-E-TC and AH65-R-TC after tissue culture passage<sup>a</sup>

5' UTR nt1-204					ORF1a gene continued					ORF1b gene nt13335-21488					S gene nt23635-27726					4.9 kDNSP gene nt27716-27805				
nt <sup>b</sup>	AH65-E	AH65-E-TC <sup>c,d</sup>	AH65-R <sup>d</sup>	AH65-R-TC <sup>d</sup>	nt <sup>b</sup>	AH65-E	AH65-E-TC <sup>c,d</sup>	AH65-R <sup>d</sup>	AH65-R-TC <sup>d</sup>	nt <sup>b</sup>	AH65-E	AH65-E-TC <sup>c,d</sup>	AH65-R <sup>d</sup>	AH65-R-TC <sup>d</sup>	nt <sup>b</sup>	AH65-E	AH65-E-TC <sup>c,d</sup>	AH65-R <sup>d</sup>	AH65-R-TC <sup>d</sup>	nt <sup>b</sup>	AH65-E	AH65-E-TC <sup>c,d</sup>	AH65-R <sup>d</sup>	AH65-R-TC <sup>d</sup>
53	C	C	T	T	7638	G	G	<b>K</b>	T	14513	G	<b>A</b>	A	A	23664	A	<b>C</b>	C	C	27746	A	<b>G</b>	G	G
ORF1a gene nt205-13356					7767	G	G	<b>R</b>	<b>R</b>	14648	T	C	T	T	23704	C	<b>G</b>	G	G	27798	C	<b>T</b>	T	T
1137	G	<b>A</b>	A	A	7947	T	T	G	G	14672	T	C	T	T	23706	T	<b>C</b>	C	C	27812	T	<b>C</b>	C	C
1209	C	<b>T</b>	T	T	7971	T	T	<b>Y</b>	C	15011	T	C	T	T	23835	T	<b>C</b>	C	C	4.8 kDNSP gene nt27883-28020				
1334	A	A	G	G	8281	C	<b>A</b>	A	A	15285	T	<b>C</b>	C	C	23971	A	<b>G</b>	G	G	27936	T	<b>C</b>	C	C
1596	C	<b>T</b>	T	T	8910	C	C	T	T	15513	A	<b>T</b>	T	T	24039	C	<b>T</b>	T	T	27943	C	<b>T</b>	T	T
2086	A	<b>R</b>	A	A	9402	T	T	C	C	16193	T	<b>C</b>	C	C	24165	T	<b>C</b>	C	C	27947	C	<b>T</b>	T	T
2224	A	<b>G</b>	G	G	9948	C	<b>T</b>	T	T	16198	T	T	G	G	24237	T	<b>C</b>	C	C	28073	T	<b>C</b>	C	C
2703	T	C	T	T	10263	G	G	A	A	16505	T	<b>G</b>	G	G	24264	T	<b>C</b>	C	C	28078	G	G	T	T
2778	A	<b>G</b>	G	G	10692	T	T	C	C	16589	T	C	T	T	24690	T	<b>C</b>	C	C	28087	C	T	C	C
2928	T	<b>G</b>	G	G	10726	C	T	C	C	17078	C	T	C	C	24786	T	<b>C</b>	C	C	12.7 kD NSP gene nt28100-28429				
3112	T	<b>C</b>	C	C	10860	C	<b>T</b>	T	T	17102	G	A	G	G	25225	G	<b>A</b>	A	A	28382	A	<b>G</b>	G	G
3251	C	T	C	C	10987	T	T	<b>Y</b>	T	17882	G	<b>T</b>	T	T	25296	C	<b>T</b>	T	T	M gene nt28685-29377				
3480	T	<b>C</b>	C	C	11155	G	G	<b>R</b>	<b>R</b>	17912	C	<b>T</b>	T	T	25305	C	<b>T</b>	T	T	28855	T	<b>C</b>	C	C
3723	T	<b>C</b>	C	C	11196	T	T	<b>Y</b>	<b>Y</b>	18374	C	<b>T</b>	T	T	25345	T	<b>C</b>	C	C	29110	T	<b>C</b>	C	C
3948	C	<b>T</b>	T	T	11259	C	C	C	<b>Y</b>	18515	T	<b>C</b>	C	C	25359	C	<b>T</b>	T	T	29215	T	T	C	C
4776	G	<b>A</b>	<b>R</b>	A	11280	C	C	T	T	18692	C	<b>T</b>	T	T	25578	T	<b>C</b>	C	C	N gene nt29387-30733				
4791	T	<b>C</b>	C	C	11282	G	G	G	<b>R</b>	19691	G	A	A	A	25884	A	<b>C</b>	C	T	29680	A	<b>G</b>	G	G
4926	C	<b>T</b>	T	T	11322	C	C	C	<b>Y</b>	20208	T	<b>G</b>	G	G	26124	C	<b>T</b>	T	C	29702	A	<b>C</b>	C	C
5097	T	<b>C</b>	C	C	11439	A	<b>G</b>	G	G	20321	A	<b>G</b>	G	G	26215	T	<b>C</b>	C	T	29823	T	<b>A</b>	A	A
5346	G	<b>A</b>	A	A	11853	A	<b>T</b>	T	T	20475	C	<b>T</b>	T	T	26718	C	<b>T</b>	T	C	30118	T	T	C	C
5624	C	<b>T</b>	T	T	12105	C	C	T	T	20828	A	<b>G</b>	G	G	26791	C	<b>A</b>	C	C	30166	G	<b>A</b>	A	A
6387	T	T	C	C	12138	C	C	T	T	20927	T	<b>G</b>	G	G	27030	C	<b>T</b>	T	T	30535	G	G	A	A
6508	T	<b>C</b>	C	C	12558	A	A	T	T	32 kD NSP gene nt21498-22334					27138	T	<b>A</b>	A	A	30543	C	C	T	T
6741	G	<b>A</b>	A	A	12609	C	C	T	T	21999	A	<b>C</b>	C	C	27156	C	<b>T</b>	T	T	30639	T	T	C	C
6917	T	T	C	C	12861	C	C	A	A	HE gene nt22346-23620					27173	A	<b>G</b>	G	G	30718	T	<b>C</b>	C	C
7023	A	A	G	G	13125	A	A	C	C	22559	G	<b>T</b>	T	T	27204	C	<b>T</b>	T	T	3' UTR nt30734-30894				
7179	C	C	T	T						22600	T	<b>C</b>	C	C	27399	A	<b>C</b>	C	C	30893	T	T	T	G
7227	T	T	A	A						22656	A	A	G	G	27531	A	<b>T</b>	T	T					
										22826	C	A	C	C	27585	T	<b>A</b>	A	A					
										23137	C	<b>T</b>	T	T	27663	C	<b>T</b>	T	T					
										23242	C	<b>T</b>	T	T	27684	T	<b>A</b>	A	A					
										23384	T	A	T	T										

<sup>a</sup>The four genomic sequences were aligned with ClustalW program in Lasergene software (DNASTAR Inc). Uniform length of 30,894 nt was achieved after trimming sequence ends. <sup>b</sup>Positions for nucleotide changes in individual genes are shown. <sup>c</sup>Nucleotides are in bold italics and boxed when AH65-E mutated to nucleotides identical to AH65-R after tissue culture passage. <sup>d</sup>The ambiguous nucleotides are in bold italics and underlined.

and enteric BCoV strains from the same animals indicates the presence of an intra-host quasispecies. These mutations were scattered throughout the entire genome, but the spike gene had higher mutation number to gene length ratios relative to most of the other gene regions (mutation  $n=31$  in spike gene out of total 123 nt differences for AH65-E/AH65-R pair and  $n=30$  out of total 149 nt differences for AH187-E/AH187-R pair). This suggests a higher selective pressure on the spike gene and supports evidence from other CoVs on the role of this protein in determining host and tissue tropism (Sanchez et al., 1992, 1999; Schickli et al., 2004; Thackray and Holmes, 2004).

### Intra-isolate quasispecies

Additionally the genomes of the uncloned wild type AH65-R and AH187-E BCoVs contained a number of sequence ambiguities, i.e. nucleotide positions which could not be

resolved by manual analysis of sequence traces or exhaustive analysis by RT-PCR and cloning. Irresolvable sequence ambiguities in otherwise clean sequence data can represent the presence of multiple RNA species present in a sample. The sequence generated during this study was derived from RT-PCR and thus represents a description of a population of viruses present in the strain at the time of purification. Sequence ambiguities were found at 7 and 84 nucleotide positions in the genomes of AH65-R and AH187-E, respectively, suggesting the existence of intra-isolate quasispecies of BCoV in the same animal.

The BCoV intra-host quasispecies can develop through pathways other than genomic RNA mutations introduced during viral replication in the host. For example, exposure to and the mixing of respiratory and enteric BCoV strains under field conditions and within a host may represent one source for mixtures of different viral populations. The possibility also

Table 2  
Nucleotide differences between the AH187-E and AH187-R strains<sup>a</sup>

nt <sup>b</sup>	AH187-E <sup>c</sup>	AH187-R
<i>ORF1a gene nt205–13356</i>		
274	G	T
576	<u>W</u>	T
747	<u>Y</u>	C
1342	<u>G</u>	C
1474	G	A
2074	C	T
2224	<u>R</u>	G
2778	<u>R</u>	G
3112	<u>Y</u>	C
3723	<u>Y</u>	C
3836	<u>K</u>	G
3843	<u>T</u>	C
3948	<u>Y</u>	T
3993	<u>Y</u>	C
4215	<u>T</u>	C
4506	<u>Y</u>	C
4749	<u>T</u>	C
4961	G	A
5487	<u>K</u>	T
5541	<u>Y</u>	C
5559	<u>C</u>	T
5721	G	T
6465	G	A
6476	C	A
6672	T	C
7317	T	C
7854	<u>Y</u>	T
7947	<u>K</u>	G
7977	<u>Y</u>	T
8223	<u>T</u>	A
8281	<u>M</u>	A
8436	<u>T</u>	C
8575	G	A
8746	<u>Y</u>	C
8982	<u>C</u>	T
9402	<u>Y</u>	C
9633	<u>C</u>	T
9948	<u>Y</u>	T
10263	<u>R</u>	A
10365	A	G
10673	<u>Y</u>	C
10860	<u>C</u>	T
11196	<u>Y</u>	T
11223	<u>T</u>	C
11259	<u>Y</u>	C
11280	<u>Y</u>	C
11308	<u>C</u>	T
11439	<u>R</u>	G
11844	C	T
12558	<u>W</u>	A
12609	<u>Y</u>	C
12729	<u>R</u>	G
12861	<u>A</u>	C
12996	A	G
13017	C	T
13091	G	A
<i>ORF1b gene nt13335–21488</i>		
13418	<u>Y</u>	T
13631	<u>R</u>	A
14429	<u>Y</u>	T
14513	<u>R</u>	A
14651	<u>W</u>	T

Table 2 (continued)

nt <sup>b</sup>	AH187-E <sup>c</sup>	AH187-R
<i>ORF1b gene nt13335–21488</i>		
14672	<u>Y</u>	C
14744	<u>T</u>	C
15020	A	G
15095	T	C
15170	T	G
15285	<u>Y</u>	C
15513	<u>W</u>	T
16193	<u>Y</u>	C
16505	<u>K</u>	G
16814	<u>T</u>	G
16847	T	C
16925	<u>Y</u>	C
17018	<u>C</u>	T
17882	<u>R</u>	G
18311	<u>R</u>	G
18479	<u>W</u>	T
18515	<u>Y</u>	C
19478	<u>C</u>	T
19556	G	A
19631	T	C
19643	A	C
20208	G	T
20687	<u>R</u>	G
20795	<u>Y</u>	T
20828	<u>R</u>	G
20927	<u>K</u>	G
21236	G	A
<i>32 kDa NSP gene nt21498–22334</i>		
21563	T	C
21662	<u>Y</u>	T
21719	<u>Y</u>	T
21842	<u>Y</u>	T
21962	<u>Y</u>	C
21999	<u>M</u>	C
22025	<u>M</u>	A
<i>HE gene nt22346–23620</i>		
22559	<u>K</u>	G
22600	<u>Y</u>	T
23056	<u>T</u>	C
23444	C	T
23446	C	T
23494	T	C
<i>S gene nt23635–27726</i>		
23704	G	C
23835	<u>Y</u>	T
23890	<u>T</u>	C
24264	<u>Y</u>	C
24426	<u>Y</u>	C
24690	<u>C</u>	T
25130	G	A
25135	T	C
25160	A	C
25163	C	G
25167	T	C
25296	<u>Y</u>	C
25305	<u>Y</u>	C
25359	<u>Y</u>	C
25484	<u>Y</u>	C
25862	<u>K</u>	T
28595	<u>G</u>	A
26215	C	T



Table 2 (continued)

nt <sup>b</sup>	AH187-E <sup>c</sup>	AH187-R
<i>S gene nt23635–27726</i>		
26718	<u><b>Y</b></u>	C
27030	<u><b>Y</b></u>	T
27138	<u><b>W</b></u>	A
27156	<u><b>Y</b></u>	T
27173	<u><b>R</b></u>	G
27204	<u><b>Y</b></u>	T
27219	<u><b>T</b></u>	C
27220	A	T
27360	<u><b>Y</b></u>	T
27531	<u><b>W</b></u>	A
27663	<u><b>Y</b></u>	T
27720	<u><b>M</b></u>	A
<i>4.9 kDa NSP gene nt27716–27805</i>		
27746	<u><b>R</b></u>	A
27812	<u><b>Y</b></u>	T
27842	<u><b>G</b></u>	T
<i>4.8 kDa NSP gene nt27883–28020</i>		
27893	C	T
27947	C	T
28044	G	A
28073	<u><b>Y</b></u>	C
28078	<u><b>K</b></u>	G
<i>M gene nt28685–29377</i>		
28716	T	A
28855	<u><b>Y</b></u>	C
29110	<u><b>Y</b></u>	C
<i>N gene nt29387–30733</i>		
29532	C	T
29680	<u><b>R</b></u>	G
29702	<u><b>M</b></u>	C
29823	<u><b>W</b></u>	A
30166	<u><b>R</b></u>	A
30718	<u><b>Y</b></u>	C
<i>3' UTR nt30734–30894</i>		
30767	<u><b>Y</b></u>	C

<sup>a</sup> The two genomic sequences were aligned with ClustalW program in Lasergene software (DNASTAR Inc). Uniform length of 30894 nt was achieved after trimming sequence ends. <sup>b</sup>Positions for nucleotide changes in individual genes are shown. <sup>c</sup>The ambiguous nucleotides are in bold italics and underlined.

exists that the calves swallowed respiratory secretions and hence respiratory viruses were introduced orally into the intestinal tract. Likewise, calves' nostrils are also possibly contaminated with enteric viruses by direct contact and inhalation of feces. Formation of intra-isolate viral quasispecies highlights the complexity of BCoV evolution under field conditions. The present study did not show a tendency of intra-isolate quasispecies formation of BCoV-E strains compared to BCoV-R strains. Although the AH187-E strain had more ambiguities than the AH65-R strain (84 nt vs. 7 nt), neither the AH65-E nor the AH187-R had any ambiguities. Our quasispecies data cannot solely be explained by potential physical contamination between respiratory and enteric sample materials because 1 and 6 nt ambiguities were also found in the AH65-E-TC and AH65-R-TC isolates, respectively after tissue

Table 3

Pair-wise comparison of divergent nucleotide positions among the six BCoVs<sup>a</sup>

nt <sup>b</sup>	AH65-E	AH65-E-TC	AH65-R	AH65-R-TC	AH187-E	AH187-R
<i>5' UTR nt1–204</i>						
53	C	C	T	T	C	C
<i>ORF1a gene nt205–13356</i>						
274	G	G	G	G	G	T
576	T	T	T	T	W	C
747	C	C	C	C	Y	C
1137	G	A	A	A	G	G
1209	C	T	T	T	C	C
1334	A	A	G	G	A	A
1342	G	G	G	G	G	C
1474	G	G	G	G	G	A
1596	C	T	T	T	T	T
2074	C	C	C	C	C	T
2086	A	G	A	A	A	A
2224	A	G	G	G	R	G
2703	T	C	T	T	T	T
2778	A	G	G	G	R	G
2928	T	G	G	G	G	G
3112	T	C	C	C	Y	C
3251	C	T	C	C	C	C
3480	T	C	C	C	T	T
3540	C	C	C	C	T	T
3723	T	C	C	C	Y	C
3836	G	G	G	G	K	G
3843	T	T	T	T	T	C
3948	C	T	T	T	Y	T
3993	T	T	T	T	Y	C
4215	T	T	T	T	T	C
4506	T	T	T	T	Y	C
4749	T	T	T	T	T	C
4776	G	A	R	A	G	G
4791	T	C	C	C	C	C
4926	C	T	T	T	C	C
4961	G	G	G	G	C	A
5097	T	C	C	C	T	T
5112	C	C	C	C	T	T
5310	C	C	C	C	T	T
5346	G	A	A	A	A	A
5487	G	G	G	G	K	T
5541	T	T	T	T	Y	C
5559	C	C	C	C	C	T
5624	C	T	T	T	C	C
5721	G	G	G	G	G	T
6303	T	T	G	G	T	T
6387	T	T	C	C	T	T
6465	A	A	A	A	G	A
6476	C	C	C	C	C	A
6508	T	C	C	C	C	C
6672	T	T	T	T	T	C
6741	G	A	A	A	A	A
6917	T	T	C	C	C	C
7023	A	A	G	G	A	A
7179	C	C	T	T	C	C
7227	T	T	A	A	T	T
7317	T	T	T	T	T	C
7638	G	G	K	T	G	G
7767	G	G	R	R	G	G
7854	T	T	T	T	Y	T
7947	T	T	G	G	K	G
7971	T	T	Y	C	T	T
7977	T	T	T	T	Y	T
8223	T	T	T	T	T	A

(continued on next page)

Table 3 (continued)

nt <sup>b</sup>	AH65-E	AH65-E-TC	AH65-R	AH65-R-TC	AH187-E	AH187-R
<i>ORF1a gene nt205–13356</i>						
8281	C	A	A	A	M	A
8436	C	C	C	C	T	C
8575	G	G	G	G	G	A
8746	T	T	T	T	Y	C
8910	C	C	T	T	C	C
8982	C	C	C	C	C	T
9402	T	T	C	C	Y	C
9633	C	C	C	C	C	T
9948	C	T	T	T	Y	T
10263	G	G	A	A	R	A
10365	A	A	A	A	A	G
10692	T	T	C	C	T	T
10726	C	T	C	C	C	C
10860	C	T	T	T	C	T
10987	T	T	Y	T	T	T
11155	G	G	R	R	G	G
11196	T	T	Y	Y	Y	T
11223	C	C	C	C	T	C
11259	C	C	C	Y	T	C
11280	C	C	T	T	Y	C
11282	G	G	G	R	G	G
11308	T	T	T	T	C	T
11322	C	C	C	Y	C	C
11439	A	G	G	G	R	G
11844	T	T	T	T	C	T
11853	A	T	T	T	T	T
12105	C	C	T	T	C	C
12138	C	C	T	T	C	C
12558	A	A	T	T	W	A
12609	C	C	T	T	Y	C
12729	G	G	G	G	R	G
12861	C	C	A	A	A	C
12996	G	G	G	G	A	G
13017	C	C	C	C	C	T
13091	A	A	A	A	G	A
13125	A	A	C	C	A	A
<i>ORF1b gene nt13335–21488</i>						
13418	T	T	T	T	Y	T
13631	A	A	A	A	R	A
14429	T	T	T	T	Y	T
14513	G	A	A	A	R	A
14648	T	C	T	T	T	T
14651	T	T	T	T	W	T
14672	T	C	T	T	Y	C
14744	T	T	T	T	T	C
15011	T	C	T	T	T	T
15020	G	G	G	G	A	G
15095	T	T	T	T	T	C
15170	T	T	T	T	T	G
15285	T	C	C	C	Y	C
15513	A	T	T	T	W	T
16193	T	C	C	C	Y	C
16198	T	T	G	G	T	T
16505	T	G	G	G	K	G
16589	T	C	T	T	C	C
16814	T	T	T	T	T	G
16847	T	T	T	T	T	C
16925	T	T	T	T	Y	C
17018	C	C	C	C	C	T
17078	C	T	C	C	C	C
17102	G	A	G	G	G	G

Table 3 (continued)

nt <sup>b</sup>	AH65-E	AH65-E-TC	AH65-R	AH65-R-TC	AH187-E	AH187-R
<i>ORF1b gene nt13335–21488</i>						
17882	G	T	T	T	R	G
17912	C	T	T	T	C	C
18311	G	G	G	G	R	G
18374	C	T	T	T	C	C
18479	T	T	T	T	W	T
18515	T	C	C	C	Y	C
18692	C	T	T	T	C	C
19478	C	C	C	C	C	T
19556	A	A	A	A	G	A
19631	T	T	T	T	T	C
19643	C	C	C	C	A	C
19691	G	A	A	A	G	G
20208	T	G	G	G	G	T
20321	A	G	G	G	A	A
20475	C	T	T	T	C	C
20687	G	G	G	G	R	G
20795	T	T	T	T	Y	T
20828	A	G	G	G	R	G
20927	T	G	G	G	K	G
21236	G	G	G	G	G	A
<i>32 kDa NSP gene nt21498–22334</i>						
21563	T	T	T	T	T	C
21662	T	T	T	T	Y	T
21719	T	T	T	T	Y	T
21842	T	T	T	T	Y	T
21962	C	C	C	C	Y	C
21999	A	C	C	C	M	C
22025	A	A	A	A	M	A
<i>HE gene nt22346–23620</i>						
22559	G	T	T	T	K	G
22600	T	C	C	C	Y	T
22656	A	A	G	G	A	A
22826	C	A	C	C	C	C
23056	T	T	T	T	T	C
23137	C	T	T	T	T	T
23242	C	T	T	T	T	T
23384	T	A	T	T	T	T
23444	C	C	C	C	C	T
23446	C	C	C	C	C	T
23494	T	T	T	T	T	C
<i>S gene nt23635–27726</i>						
23664	A	C	C	C	A	A
23704	C	G	G	G	G	C
23706	T	C	C	C	T	T
23835	T	C	C	C	Y	T
23890	T	T	T	T	T	C
23971	A	G	G	G	A	A
24039	C	T	T	T	C	C
24165	T	C	C	C	T	T
24237	T	C	C	C	T	T
24264	T	C	C	C	Y	C
24426	C	C	C	C	Y	C
24690	T	C	C	C	C	T
24786	T	C	C	C	T	T
25130	G	G	G	G	G	A
25135	T	T	T	T	T	C
25160	A	A	A	A	A	C
25163	C	C	C	C	C	G
25167	T	T	T	T	T	C

Table 3 (continued)

nt <sup>b</sup>	AH65-E	AH65-E-TC	AH65-R	AH65-R-TC	AH187-E	AH187-R
<i>S gene nt23635–27726</i>						
25225	G	A	A	A	G	G
25296	C	T	T	T	Y	C
25305	C	T	T	T	Y	C
25345	T	C	C	C	T	T
25359	C	T	T	T	Y	C
25484	C	C	C	C	Y	C
25578	T	C	C	C	T	T
25862	G	G	G	G	K	T
25884	A	C	C	C	C	C
25895	G	G	G	G	G	A
26124	C	T	T	T	C	C
26215	T	C	C	C	C	T
26718	C	T	T	T	Y	C
26791	C	A	C	C	C	C
27030	C	T	T	T	Y	T
27138	T	A	A	A	W	A
27156	C	T	T	T	Y	T
27173	A	G	G	G	R	G
27204	C	T	T	T	Y	T
27219	T	T	T	T	T	C
27220	A	A	A	A	A	T
27360	T	T	T	T	Y	T
27399	A	C	C	C	A	A
27531	A	T	T	T	W	A
27585	T	A	A	A	T	T
27663	C	T	T	T	Y	T
27684	T	A	A	A	T	T
27720	A	A	A	A	M	A
<i>4.9 kDa NSP gene nt27716–27805</i>						
27746	A	G	G	G	R	A
27798	C	T	T	T	C	C
27812	T	C	C	C	Y	T
27842	G	G	G	G	G	T
<i>4.8 kDa NSP gene nt27883–28020</i>						
27893	C	C	C	C	C	T
27936	T	C	C	C	T	T
27943	C	T	T	T	C	C
27947 <sup>c</sup>	<u>C</u>	<u>T</u>	<u>T</u>	<u>T</u>	<u>C</u>	<u>T</u>
28044	G	G	G	G	G	A
28073	T	C	C	C	Y	C
28078	G	G	T	T	K	G
28087	C	T	C	C	C	C
<i>12.7 kDa NSP gene nt28100–28429</i>						
28382	A	G	G	G	G	G
<i>M gene nt28685–29377</i>						
28716	A	A	A	A	T	A
28855	T	C	C	C	Y	C
29110	T	C	C	C	Y	C
29215	T	T	C	C	T	T
<i>N gene nt29387–30733</i>						
29532	C	C	C	C	C	T
29680	A	G	G	G	R	G
29702	A	C	C	C	M	C
29823	T	A	A	A	W	A
30118	T	T	C	C	T	T
30166	G	A	A	A	R	A
30535	G	G	A	A	G	G
30543	C	C	T	T	C	C

Table 3 (continued)

nt <sup>b</sup>	AH65-E	AH65-E-TC	AH65-R	AH65-R-TC	AH187-E	AH187-R
<i>N gene nt29387–30733</i>						
30639	T	T	C	C	T	T
30718	T	C	C	C	Y	C
<i>3' UTR nt30734–30894</i>						
30767	C	C	C	C	Y	C
30893	T	T	T	G	T	T

<sup>a</sup>The six genomic sequences were aligned with ClustalW program in Lasergene software (DNASTAR Inc.). Uniform length of 30894 nt was achieved after trimming sequence ends. <sup>b</sup>Positions for nucleotide changes in individual genes are shown. <sup>c</sup>The common mutation from enteric to respiratory strains at nucleotide position 27947 is in bold italics and underlined.

culture passage. The single ambiguity at nucleotide position 2086 of the AH65-E-TC apparently occurred after tissue culture adaptation and plaque purification (Table 1). Similarly, ambiguous positions at nucleotides 11,259, 11,282, and 11,322 of AH65-R-TC were observed after tissue culture passage and plaque purification. The intra-host BCoV genetic diversities and the intra-isolate ambiguities discovered in the tissue culture BCoV isolates indicate that the mixtures of genetically different viruses were formed intrinsically during viral replication within the host or developed after tissue culture adaptation.

### Changes after tissue culture adaptation

The AH65-E-TC isolate showed 104 nt mutations from the parental AH65-E strain after 15 passages in cell culture plus 1 plaque purification. In contrast, the genome of the AH65-R-TC isolate changed at only 8 nucleotide positions from the AH65-R strain after a similar number of cell culture passages (14 tissue culture passages and 1 plaque purification). These findings suggest that enteric BCoV strains are more prone to change than respiratory BCoV strains during cell culture adaptation and passage. Unexpectedly, of the 104 mutations observed in the AH65-E-TC, 86 mutated to be the same as the corresponding nucleotides of the AH65-R isolate (Table 1). As a result, the AH65-E-TC genetically resembled the AH65-R strain more than the parent AH65-E strain. Particularly noteworthy, whereas the spike gene of the AH65-E differed from that of the AH65-R at 31 nucleotide positions, there was only one nucleotide difference in spike genes between the AH65-E-TC isolate and the AH65-R strain (Table 1). This is surprising because the HRT-18 cells used for *in vitro* viral adaptation and passage are human rectum epithelial cells and therefore are of intestinal and not respiratory origin. However, the AH65-E-TC isolate still differed from the AH65-R strain at 48 nucleotides with 37 of these divergences located in ORF1a and 1b genes. The latter substantial genomic differences argue against the AH65-E-TC isolate being an AH65-R variant that arose from contamination during the process of tissue culture adaptation and passage. The data from these isolates suggest that similar evolutionary processes may have occurred *in vitro* during BCoV adaptation to cell culture as those happening *in vivo* during adaptation from enteric to respiratory tropism. Only the AH65-E-TC and AH65-R-TC pair was selected initially for sequencing to see if they



will be any significant mutations in the genome of enteric and respiratory BCoV strains after tissue culture passage. Given the interesting trend of mutations observed in the AH65-E-TC and AH65-R-TC genomes, sequencing of the other enteric and respiratory BCoV pair (e.g. AH187-E-TC and AH187-R-TC) will be attempted in the future to confirm if similar mutations occur in AH187-E-TC that mimic the AH187-R strain sequence. However, differences in the number and trend of mutations between AH187-E/AH187-R and AH65-E/AH65-R strains after tissue culture passage are expected based on the divergence of their genomes. In addition, it is possible that certain BCoV strains may require more cumulative passages in tissue culture than others to achieve significant genetic mutations, tropism shifts, or changes in virulence. Nevertheless, our observation that BCoV-E gained genetic mutations resembling the sequence of BCoV-R after tissue culture passage is consistent with that found for other members of the coronavirus family. For example, transmissible gastroenteritis virus (TGEV) strains after 60 to 115 continuous tissue passages may lose their enteric tropism and gain respiratory tropism (Harada et al., 1969; Zhang et al., 2007).

Until now, it has been unclear whether nasal and rectal isolates of BCoVs are the same virus or whether BCoV undergoes significant genetic changes during adaptation to different tissues of the host. In general, biological, antigenic, and genetic differences were found between BCoV-R and BCoV-E (Gelinas et al., 2001a, 2001b; Hasoksuz et al., 1999; Lin et al., 2002). However, other investigators did not find consistent differences in biologic properties between BCoV-R and BCoV-E strains (Reynolds et al., 1985; Zhang et al., 1994a). The inconsistent results are partially ascribed to the fact that the BCoV strains compared varied widely in origin or year of isolation; for example, comparisons between BCoV-R and BCoV-E strains have often been compromised when viruses were isolated from different animals at different times (Reynolds et al., 1985). To accurately define tropism determinants of BCoV-R and BCoV-E, a systematic investigation of the complete genome sequences of BCoV-R and BCoV-E strains isolated concurrently from the same animals and examined both prior to and after cell culture adaptation is necessary.

Only in recent years, has the genetic diversity of BCoV within the same animal been studied, but either only partial genomes were sequenced or the field BCoV isolates were first adapted to cell culture before sequencing. To our knowledge, this is the first report on sequencing and comparison of the full genomes of the original wild type paired animal BCoV specimens that fully describe the composition and complexity of BCoV intra-host evolution and the first to fully describe the existence of BCoV quasispecies. Chouljenko et al. (2001) sequenced a paired respiratory (LUN strain) and enteric (ENT strain) BCoV from the same animal with fatal shipping fever pneumonia and they found 107 nucleotide variations throughout the genomes between these two viruses. However, in that study the original BCoV samples were subjected to multiple cell culture passages and plaque purifications before sequencing. Such manipulations have the potential to cause genetic changes in BCoV genomes as evidenced in our study. In addition, no

nucleotide ambiguities in the genomes were reported in that study possibly because the BCoVs were plaque purified multiple times and were sequenced immediately after plaque purification.

Previously, the S1 subunit of the spike gene of the tissue culture passaged and plaque purified AH65-E and AH65-R (8 passages for AH65-E and 6 passages for AH65-R) and AH187-E and AH187-R (2 passages for AH187-E and 3 passages for AH187-R) pairs were analyzed in our lab (Hasoksuz et al., 2002b). In that study, the S1 protein was found almost identical to each other between the concurrent enteric and respiratory strains except for one and two amino acid differences identified for the AH65-E/AH65-R pair and the AH187-E/AH187-R pair, respectively. Those findings are consistent with the observations in the present study that the S gene sequence of the AH65-E strain after tissue culture adaptation and passage closely resembled that of the AH65-R strain. However, discrepancies in amino acid residues at certain positions were found in the S1 region of previous sequences and present sequences and no nucleotide ambiguity positions were observed in that region in the previous study. The sequence discrepancy and lack of ambiguity positions likely reflect the fact that the viruses in our previous study were at different cell culture passage levels and were sequenced immediately after the plaque purification.

It is widely speculated that the spike protein of CoV is the major determinant for tropism. The spike gene of TGEV was identified as the determinant for enteric tropism (Sanchez et al., 1999). Mutations at two nucleotides, nt positions 214 and 655, in the spike gene of TGEV can potentially cause a shift from enteric to respiratory tropism (Ballesteros et al., 1997). We did not observe mutations at nt positions 214 and 655 in the spike gene (corresponding to nt 23,848 and 24,289 in the genome, respectively) of AH65-E/AH65-R and AH187-E/AH187-R strains, suggesting that determinants for enteric to respiratory tropism shifts may be different between the group 1 TGEV CoV and the group 2 BCoVs. Chouljenko et al. (1998) proposed amino acid 769 immediately downstream of the cleavage site (764–768) and amino acid 1026 of the spike protein as two critical residues responsible for respiratory tropism of BCoV. In our BCoV-E and BCoV-R pairs, no amino acid difference was found at these two positions, suggesting that the amino acid positions 769 and 1026 are not relevant to tropism shifts for our strains. Our sequence analysis cannot pinpoint specific amino acid residue determinants for tropism because the enteric strains differed from respiratory strains at multiple positions. Analysis of the two pairs of viruses did not identify many common genetic changes between BCoV-E and BCoV-R strains except for a shared C to T mutation at nt 27,947 resulting in aa T to I mutation in the 4.8 kDa non-structural protein. This mutation was not among the 107 genomic mutations observed between the respiratory and enteric BCoV pair LUN/ENT (Chouljenko et al., 2001). Further study is needed to verify the exact function of this mutation by reverse genetics and animal studies. However, it is likely that for BCoV tissue tropism, shifts are determined by multiple factors involving a combination of

genetic mutations. Others observed a truncated 4.9 kDa non-structural protein of 29 aa in BCoV-R strains instead of 43 aa for BCoV-E strains and suggested that this protein could be involved in determining respiratory tropism (Gelinas et al., 2001b; Vijgen et al., 2006). But this hypothesis also has not been experimentally proven. Our sequence data indicated that all respiratory and enteric BCOVs have the truncated 4.9 kDa non-structural protein of 29 aa, suggesting that truncation of this protein is not necessarily needed for enteric to respiratory tropism shift. Involvement of mutations in this protein in tropism shift remains to be verified.

Our analysis has defined the presence of multiple BCoV species present in the same animal. We detected evidence for intra-host quasispecies (significant sequence variation between enteric and respiratory strains) and also intra-isolate quasispecies (sequence ambiguities within the same strains). The comprehensive dissection of BCoV quasispecies will aid in an understanding of BCoV population dynamics and pathogenesis. The presence of intra-host and intra-isolate BCoV quasispecies suggests that BCoV evolves through selection pressures working on a quasispecies basis. Recent evidence has suggested that many RNA viruses exist as quasispecies *in vivo* and *in vitro* (Domingo et al., 1998). Viral quasispecies are characterized by continuous genetic variation within virus populations which is a result of the high error rates of most RNA virus-encoded RNA-dependent RNA polymerases. Quasispecies evolution can occur in individual hosts, geographically related areas, or different temporal phases during the course of infection. Examples of RNA viruses with distinctive quasispecies include bovine viral diarrhea virus, West Nile virus, and hepatitis A virus (Costa-Mattioli et al., 2006; Jerzak et al., 2005; Jones et al., 2002). Rapid evolutionary change allows viruses to adapt to changing micro or macro environments, increase viral fitness, and maintain balance with host immune systems in order to achieve long-term survival in nature. It is believed that pathogenesis is determined by viral quasispecies rather than by the action of a single genotype (Vignuzzi et al., 2006). Increasing evidence indicates that quasispecies development may lead to the selection of virulent viruses and to the emergence of new viral species (Domingo, 1997; Domingo et al., 2006; Holland et al., 1982). Research suggests that SARS-CoV, a newly emerging human CoV, exists as quasispecies in individual patients (Xu et al., 2004) and a genetically diverse population of SARS-like coronaviruses are present in geographically closely related Chinese horseshoe bats (Ren et al., 2006).

Understanding BCoV evolution is important because not only is this virus of economical importance to the cattle industry, but also this virus shows antigenic and biological similarities to other group 2a human CoVs including HCoV-OC43 and shares biological, pathogenic properties, and pneumoenteric tropisms with group 2b SARS-CoVs. Based on sequence analysis, researchers have shown the potential zoonotic transmission of a BCoV to human beings (Vijgen et al., 2005) and previously confirmed the isolation of a bovine-like CoV from a child with diarrhea (Zhang et al., 1994b). Moreover the bovine-like, human CoV isolate infected and

caused disease in gnotobiotic calves (Han et al., 2006). Information on BCOVs genomics will aid in our understanding of the evolution of both BCOVs and human CoVs including HCoV-OC43 and the recently emerged SARS-CoV.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.03.018.

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