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Nucleotide and Predicted Amino Acid Sequences of All Genes Encoded by the 3' Genomic Portion (9.5 kb) of Respiratory Bovine Coronaviruses and Comparisons Among Respiratory and Enteric Coronaviruses

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Abstract. The 3'-ends of the genomes (9538 bp) of two wild-type respiratory bovine coronavirus (RBCV) isolates LSU and OK were obtained by cDNA sequencing. In addition, the 3'-end of the genome (9545) of the wild-type enteric bovine coronavirus (EBCV) strain LY-138 was assembled from available sequences and by cDNA sequencing of unknown genomic regions. Comparative analyses of RBCV and EBCV nucleotide and deduced amino acid sequences revealed that RBCV-specific nucleotide and amino acid differences were disproportionately concentrated within the S gene and the genomic region between the S and E genes. Comparisons among virulent and avirulent BCV strains revealed that virulence-specific nucleotide and amino acid changes were located within the S and E genes, and the 32 kDa open reading frame.

Key words: cDNA, S, non-structural, structural, respiratory, coronavirus

Introduction

Coronaviruses are important etiological agents of human and animal diseases including respiratory infection, gastroenteritis, hepatic and neurological disorders as well as immune-mediated disease such as feline infectious peritonitis, and other persistent infections (1,2). Enteric bovine coronaviruses (EBCV) are generally associated with enteric disease of newborn calves and winter dysentery of adult cattle (2). Recently, numerous respiratory bovine coronaviruses (RBCV) were isolated in our laboratory from cattle arriving with fever and respiratory disease in feedlots or livestock shows of 8 different states in the USA. The cytopathogenic, cell fusion, and other phenotypic properties of these viruses were different from the known EBCV (3).

Coronaviruses contain a single stranded, capped, and polyadenylated positive-sense (infectious) RNA molecule of approximately 30 kb length, which directs the synthesis of a nested set of subgenomic mRNAs (4,5). The 3'-end of the genomic RNA consists of approximately 9.5 kb and contains the spike (S) glycoprotein, the hemagglutinin-esterase (HE) glycoprotein, the integral membrane (M) protein, the small membrane protein (E) and the phosphorylated nucleocapsid (N) protein and a number of ORFs potentially encoding non-structural proteins (N_s) (5). The 32 kDa non-structural protein is a phosphoprotein that accumulates in the cytoplasm of infected cells (6,7). It is not known whether the 12.7 and 4.8 kDa ORFs are expressed in infected cells, while the 4.9 kDa putative protein, most likely, is not translated (8).

BCV uses N-acetyl-9-O acetyl neuraminic acid as receptor determinant to initiate infection (9). Although the HE glycoprotein also has an affinity for 9-O-acetylated sialic acid, the S glycoprotein was identified as the major sialic acid binding protein of BCV (10). The S glycoprotein facilitates viral

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The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession number AF058942, AF058943, AF058944.

attachment to susceptible cells, causes cell fusion after cell-surface expression (fusion from within), and induces viral infectivity neutralizing antibodies (1). Porcine transmissible gastroenteritis virus (TGEV) strains were isolated which exhibited respiratory tissue tropism. These viruses contained point mutations or deletions within the first 250 aa of TGEV S1 which were associated with reduced enteropathogenicity and loss of hemagglutinating activity (11–13).

To examine the genetic basis for the phenotypic differences between RBCV and EBCV, we cloned and sequenced the 3'-end of the viral genomes of two virus strains RBCV-LSU-94LSS-051-2(LSU) and RBCV-OK-0514-3(OK) that originated from Louisiana and Oklahoma cattle, respectively. We report here, the nucleotide and predicted amino acid sequences of all genes encoded by the 3' genomic portion (9.5 kb) of two wild-type RBCV strains and comparisons among respiratory and enteric coronaviruses.

Materials and Methods

Viruses and cell line. All RBCV and EBCV strains were propagated in the G clone of human rectal tumor cells (HRT-18G) developed recently through selection and medium modulation (3). Supernatant fluids from infected HRT-18G cells were collected and viruses were purified as described (14). RBCV OK and LSU virus stocks were tested at the third and fourth passages, respectively. EBCV LY-138(LY) virus stocks were prepared at the second passage, while the EBCV-L9 virus strain, derived from the EBCV-Mebus strain, had been propagated 80 times in cell cultures.

Strategy for cDNA construction and assembly of the 9.5 Kb cDNA sequence representing the 3'-end of different BCV strains. TRI Reagent from the Molecular Research Center, Inc. (Cincinnati, OH, USA), was used for total RNA extraction. Ready-To-Go You-Prime First-Strand Beads from Pharmacia Biotech Inc. (Uppsala, Sweden) were used for cDNA library construction. All amplifications were performed using the Gene-Amp PCR system 9600 (Perkin-Elmer, Norwalk, CT, USA) with PCR reagents and AmpliTaq from Perkin-Elmer. The TA-cloning kit from Invitrogen Inc. (San Diego, CA) was used for cloning of RT-PCR products. Restriction

enzymes were obtained from New England Biolabs (Beverly, MA, USA).

The 3' genomic end of BCV Mebus strain consisting of 8695 nucleotides was assembled from available sequences deposited in Genbank. The accession numbers of the cDNA sequences used to assemble the EBCV Mebus genome were M31053 for S and HE genes, M31054 for the 4.9, 4.8, 12.7 and 9.5 kDa (E) ORFs, and M16620 for the M, N genes and I ORF. The assembled Mebus genomic sequence did not contain the 32 kDa ORF. An 850 nt sequence containing the 32 kDa ORF of BCV-Quebec (accession number X15445) was used for comparisons with other BCV. The S and HE cDNA sequences specified by LY-138 were previously reported (15,16).

A series of overlapping cDNA clones representing the entire 3'-end of two RBCV isolates and unpublished sequences of LY-138 were constructed. Two cDNA libraries were produced, a library was made using the BCV3' primer representing the 3' terminus of the genomic RNA, and a second cDNA library was produced using an oligonucleotide (3B11) to prime cDNAs starting at nucleotide 6345 (counting from the 3'-end of the viral genome) (Fig. 1). The entire 9545 nt sequence representing the 3'-end of the BCV genome was divided into six overlapping cDNA regions. Each cDNA was amplified by PCR using specific primer pairs. Primer pair 5F6/BCV 3' amplified a cDNA fragment containing the M and N genes. Primer pair 5F5/3B3 amplified a cDNA fragment containing the 3'-end of S, 4.9 kDa, 4.8 kDa, 12.7 kDa, E, M and the 5'-end of N genes. Primer pair B5'/B3' amplified the 3'-end of the spike gene, primer pair 5F24/A3' amplified a cDNA that coded for the carboxy-terminal portion of the S1 subunit, primer pair A5'/3B11 amplified a cDNA fragment that coded for the amino-terminus of S, and primer pair 5F16/3B10 amplified the 32 kDa and HE genes. B3' and A5' primers were designed to contain an extra *Bam*HI and *Eco*RI sites for cloning purposes, while a *Bst*XI site was naturally present in the 5F24 primer. The actual primer sequences are:

BCV3':5'GTGATTCTTCCAATTGGCC;
B5':5'TCTTCGACAGCTGCAACC;
A3':5'GGTTGCAGCTGTCGTGAAAGA;
B3':5'AACGGATCCAATATATCGTCAGGAGCC;
A5'-5':GCTGAATTCGATAATGGTACTAGGCTG;
5F16-5':AGAATGGCAGTTGCTTATG;
5F24-5':TCT-CCATTAAATTGGGAACGTAAG;
3B10-5':GATTATGGTCTAAGCATCATG;

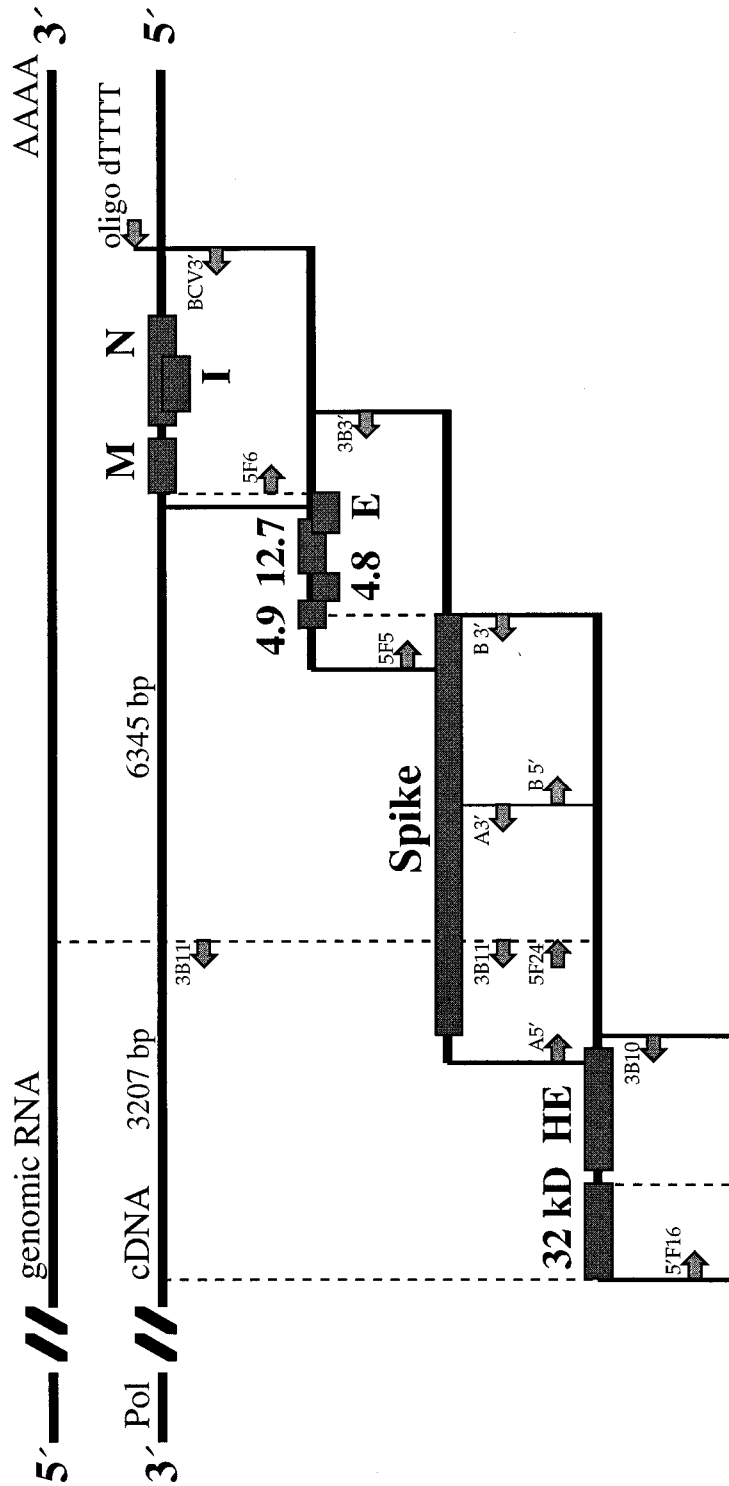


Fig. 1. BCV genome arrangement and cDNA cloning and sequencing strategy. Top line depicts the + stranded RNA genome of BCV with a truncation marking (//) separating the 5' portion of the genome coding for the RNA-dependent RNA polymerase (Pol) from the remaining BCV genes. The second line diagrams two cDNA libraries produced by reverse transcription of the BCV genome using oligonucleotide primers BCV3' and 3B11. Coding sequences for each gene are indicated as filled rectangular boxes. The approximate location of oligonucleotide primers used for RT-PCR and cloning of individual cDNAs are shown as arrows pointing towards the direction of cDNA synthesis.

3B11-5':GGTCTTACGTTCCCAATTTAATGG;
 5F5-5':GGTATAGCCCCTAAGAGTG;
 5F6-5':GTTTAATAGAGGTAGGCAG;
 3B3-5':GGTAGCTGAGAAGTAGCAG.

DNA sequencing and analyses. DNA sequencing was carried out with the modified dideoxynucleotide chain termination procedure (17) using Sequenase™ from Amersham Life Science (Arlington Heights, IL). All sequencing data were based on sequences obtained from at least two independently cloned cDNA fragments. DNA sequences were analyzed with the aid of the Sequence Analysis Software Package of the Genetics Computer Group of the University of Wisconsin and the MacVector Software (IBI, New Haven, CT). Sequence data obtained from all overlapping cDNA clones were compiled using the program AssemblLine of the MacVector™ software package running on a Power Macintosh Computer. Consensus sequences of 9538 bp were produced for LSU and OK strains, and 9545 bp for the LY-138 strain (Genbank accession numbers AF058942, AF058943, AF058944 respectively). Analysis of the cDNA sequence data using the MacVector software package revealed 10 major ORFs: ORF1 (32 kDa, 15–851), ORF2 (HE, 863–2137), ORF3 (S, 2152–6243), ORF4 (4.9 kDa, 6233–6364), ORF5 (4.8 kDa, 6400–6537), ORF6 (12.7 kDa, 6617–6946), ORF7 (E, 6933–7187), ORF8 (M, 7202–7894), ORF9 (N, 7904–9250), and ORF10 (I, 7965–8588) for LSU and OK strains. The corresponding locations of ORFs starting from 4.9 kDa ORF were different for LY-138, because of a codon insertion in the 4.9 kDa ORF of LY-138 and the four nucleotide deletion in the intergenic region between 4.8 and

12.7 kDa ORFs of RBCV. The locations for these ORFs were: ORF4 (4.9 kDa, 6233–6367), ORF5 (4.8 kDa, 6403–6540), ORF6 (12.7 kDa, 6624–6953), ORF7 (E, 6940–7194), ORF8 (M, 7209–7901), ORF9 (N, 7911–9257), and ORF10 (I, 7972–8595).

Results

Overall comparisons of genes and predicted proteins specified by RBCV (LSU, OK) and EBCV (LY, Mebus) strains. To establish the close evolutionary relationship between LSU and OK strains and to ascertain RBCV-specific amino acid changes (conserved in LSU and OK but different in other strains), a pairwise comparison of nucleotide and amino acid differences among BCV strains for all ORFs, except for the ORF coding for the RNA-dependent-RNA-polymerase and the 32 kDa protein was performed (Table 1). In general, the nucleotide and amino acid sequences of RBCV strains LSU and OK were more conserved to each other than to EBCV strains LY-138 and Mebus, and they were more divergent to the Mebus strain than to the LY-138. Specifically, the amino acid sequence of M specified by LSU and OK were identical, while they were different by one, and two aa from that of Mebus and LY-138, respectively. The S glycoprotein specified by LSU differed by only 4 amino acids from that of OK, while S glycoproteins of LSU and OK differed by 22 and 33 amino acids in comparison with the LY and Mebus S sequences, respectively. Furthermore, LSU and OK sequences of the N and I ORF (located within N) were more conserved to each other than to any other strain compared. Most amino

Table 1. The nucleotide and amino acid changes for each ORF of different BCV strains

Strain	Compared to Strain	HE 1272 (424)*	S 4089 (1363)	4.9 kDa 129 (43)	4.8 kDa 135 (45)	12.7 kDa 327 (45)	E 252 (84)	M 690 (230)	N 1344 (448)	I-ORF 621 (207)
OK	LSU	11 (4) [#]	29 (4)	2 (2)	5 (4)	3 (2)	0	1 (0)	4 (2)	3 (2)
LY	LSU	23 (5)	68 (22)	7 (4)	8 (6)	4 (1)	1 (0)	6 (2)	16 (4)	9 (6)
	OK	20 (5)	64 (22)	7 (4)	8 (5)	5 (3)	1 (0)	7 (2)	12 (4)	6 (4)
Mebus	LSU	24 (6)	70 (33)	3 (3)	8 (5)	5 (1)	2 (1)	4 (1)	17 (5)	10 (8)
	OK	23 (6)	66 (33)	3 (3)	7 (4)	6 (3)	2 (1)	5 (1)	13 (3)	7 (6)
	LY	13 (3)	57 (26)	4 (1)	5 (2)	1 (0)	1 (1)	2 (1)	9 (3)	5 (4)

* indicate the total number of nucleotides and amino acids for each gene.

[#] comparisons show the total number of nucleotide and amino acid substitutions (in parenthesis) for each ORF.

acid changes within HE, 4.9, 4.8, 12.7 kDa ORFs, E, N and the I ORF were strain-specific. HE and M contained one RBCV-specific aa change, and N and I ORF contained two RBCV-specific aa changes each.

RBCV-specific amino acid substitutions within S. The S1 subunit contained most of the RBCV-specific aa substitutions and included an amino acid change within the signal sequence as well as two clusters of amino acid substitutions within the amino-terminus and the hypervariable region (Fig. 2). The proteolytic cleavage site that separates S1 and S2 subunits was conserved among RBCV, LY-138 and Mebus strains. In contrast to the high number of RBCV-specific substitutions within S1, S2 contained only two RBCV-specific amino acid changes, an Ala 769 to Ser change immediately adjacent to the proteolytic cleavage site and an Asp 1026 to Gly located within the heptad repeat sequence. The RBCV-G95 strain was isolated from a nasal sample of a calf that had diarrhea and signs of respiratory distress (18). The nucleotide and predicted primary structures of S and HE glycoproteins specified by RBCV-G95 were reported previously (19,20). LSU and OK had ten unique aa substitutions within S in comparison to all other BCV strains, while G95, LSU, and OK shared only three aa substitutions at aa 100, aa 465, and aa 1026 (Fig. 2).

RBCV-specific nucleotide and amino acid substitutions within the 4.9, 4.8, and 12.7 kDa ORFs. The human coronavirus strain OC43 (HCV-OC43) lacks two ORFs which potentially encode two non-structural proteins of 4.9 and 4.8 kDa (21). Furthermore, the same genomic areas are deleted in three Hemagglutinating Encephalomyelitis Virus (HEV) strains of swine (22). The fact that respiratory HCV-OC43 and EBCV strains show remarkable genomic and protein similarities as well as immunological cross-reactivities, prompted us to compare the nucleotide sequences specified by the genomic region between the S and the 12.7 kDa ORF of EBCV, RBCV, HCV-OC43, and three porcine HEV strains (Fig. 3). LSU and OK contained a deletion (TAAT) in comparison with Mebus as well as LY-138. This deletion was located 31–34 bp upstream from the AUG of the 12.7 kDa ORF in the genomic region between the 4.8 kDa and 12.7 kDa ORFs. The nucleotide sequences downstream (3') to this deletion

were highly conserved among all strains. The 12.7 kDa ORF of LSU and OK contained the aa substitution of Lys 5 to Arg, while the aa substitutions of Ile 8 to Thr, and Glu 21 to Asp were unique for OK.

Comparison of the Mebus and L9 strains against virulent (LSU, OK, LY, F15) strains. To examine the evolutionary relationship of virulent strains to the avirulent, cell adapted strains, and to delineate specific amino acids which may cause loss of virulence, a comparison was performed between L9, Mebus and all other strains. The E protein which is encoded by the 9.5 kDa ORF contained one aa substitution (Gly 53 to Val) which was conserved for all virulent strains (F15, LY-138, OK, LSU) in comparison to the avirulent Mebus and L9 strains. Two other RBCV strains from Texas and Arizona cattle also contained the same aa substitution (data not shown). Four aa changes in S1 at aa 33, aa 40, aa 278 and aa 470 and three aa substitutions in S2 at aa 965, aa 1241 and aa 1341 were conserved in all virulent strains in comparison to Mebus and L9 (Fig. 2) as well as Norden Vaccine, and EBCV-Quebec strains (data not shown). The 32 kDa ORF codes for a non-structural phosphoprotein that accumulates in the cytoplasm of infected cells (6,7). The EBCV-Quebec sequence of the 32 kDa ORF was used for comparison, because the 32 kDa nucleotide sequence of the Mebus strain was not available. DNA sequencing of three independently derived cDNA clones for each strain revealed the presence of a single nucleotide (A) insertion at position 757 (aa 253) specified by both LSU and OK altering the translational reading frame for the next 9 aa until a double nucleotide insertion (AA) at nucleotides 784 and 785 restored the original frame. LY-138 specified a similar 9 aa peptide which contained one aa change, Ser to Phe at aa 254 in comparison to RBCV. In contrast, the corresponding genomic region of the avirulent EBCV-Quebec strain specified an 8 aa sequence which was different than the 9 aa sequence specified by virulent EBCV and RBCV strains. Two additional amino acid changes, Ser (192) to Ile and Pro (aa 273) to Ser (274), located at the carboxy terminal portion of the 32 kDa ORF, were specific for all virulent strains (Fig. 4). Identical aa changes were also found in the 32 kDa protein of two more RBCV strains isolated from Texas and Arizona cattle (data not shown).

	1	11	13	33	36	38	40	88	100	113	115	118	169	173	179	181	248	253	256	331	362	440	447
1. F15	M	M	L	V	I	T	T	T	I	I	H	M	N	H	R	V	M	S	M	C	S	L*	I
2. LY	M	M	L	V	V*	T	T	T	I	I	N	M	H	H	R	I*	M	S	M	C	S	V	T
3. G95	M	M	F	V	I	R*	T	R	T*	T	H	M	N	H	R	V	M	S	M	G*	S	V	T
4. LSU	M	T	F	V	I	T	T	T	I	I	D	K	N	N	Q	V	M	S	L*	C	S	V	T
5. OK	M	T	F	V	I	T	T	T	I	I	D	K	N	N	Q	V	M	S	M	C	S	V	I
6. L9	M	M	F	A	I	T	I	R	I	I	K	M	H	H	K	V	L	S	M	C	C*	V	T
7. Mebus	M	M	F	A	I	T	I	R	I	I	K	M	H	H	K	V	L	N*	M	C	S	V	T

	458	465	470	484	499	501	509	510	531	543	571	578	580	591	608	648	651	707	742	744	767	768	769
1. F15	S	V	D	S	N	P	N	S	N	S	Y	T	Q	S	D	A*	T	I	A*	V	V*	R	A
2. LY	S	V	D	S	S	S	N	S	N	S	Y	T	Q	S	D	V	T	I	S	V	R	R	A
3. G95	S	A	D	S	T	P	T*	S	D	S	H*	T	Q	A*	G*	V	T	I	S	A	R	R	A
4. LSU	S	A	D	S	S	S	N	T	G	A	Y	S	Q	S	D	V	T	F*	S	A	R	R	S
5. OK	S	A	D	S	S	S	N	T	G	A	Y	S	R*	S	D	V	T	I	S	A	R	R	S
6. L9	S	V	H	S	N	P	N	S	D	S	Y	T	Q	S	D	V	P*	S	S	A	R	R	A
7. Mebus	P*	V	H	T*	N	P	N	S	N	S	Y	T	Q	S	D	V	T	I	S	A	R	R	A

	778	817	834	906	909	927	936	965	984	988	1026	1085	1095	1100	1223	1241	1269	1286	1316	1341	1363
1. F15	N	T	C*	E*	K	A	N	E	W	A	D	R*	F*	A	V	P	T	L*	F	K	D
2. LY	N	I*	Y	D	K	A	N	E	W	A	D	A	L	A	V	P	T	V	L*	K	D
3. G95	N	T	Y	D	R*	S*	Y*	E	W	A	G	A	L	A	V	P	N*	V	F	K	D
4. LSU	N	T	Y	D	K	A	N	E	W	A	G	A	L	A	V	P	T	V	F	K	D
5. OK	N	T	Y	D	K	A	N	E	W	A	G	A	L	A	V	P	T	V	F	K	D
6. L9	N	T	Y	D	K	A	N	V	W	A	D	A	A	A	A*	H	T	V	F	I	D
7. Mebus	T*	T	Y	D	K	A	N	V	L*	V*	D	A	A	V*	V	H	T	V	F	I	D

Discussion

Genetic comparisons among different BCVs revealed substantial differences between RBCV and EBCV strains principally within the S gene and within ORFs located between the S and E genes. Furthermore, genetic differences between virulent and avirulent strains were identified within the S gene, the E gene and the 32kDa ORF. The salient features of genetic differences between RBCV and EBCV strains are discussed below:

RBCV-specific genetic alterations in the S gene. A pairwise alignment of TGEV and MHV S aa sequences revealed that the N-terminal portion of S1 which is deleted in the porcine respiratory coronaviruses (PRCV) and HCV-229E, in comparison with TGEV, is the region corresponding to the MHV receptor binding-site (aa 1–330) (23). The TGEV receptor-binding site is in a different location (aa 500–700) and aligns with the S polymorphic region of the MHV strain. Recently, it was shown that only two aa changes at the N-terminus of TGEV S resulted in the loss of enteric tropism (13). The S1 amino terminus specified by RBCV strains LSU and OK contained aa changes at aa11, aa115, aa118, aa173 and aa179 which may affect S1-mediated receptor binding. Hemagglutination of chicken red blood cells (RBC) was shown to be mediated by the S glycoprotein, because purified S of the EBCV Mebus strain agglutinated chicken RBC, while purified HE did not (10,24). RBCV strains LSU and OK agglutinated mouse and rat, but not adult chicken RBC (3). Therefore, aa changes within S specified by RBCV may be responsible for the inability to hemagglutinate chicken RBC.

The S1A virus neutralizing (VN) immunoreactive epitope (aa 351–403) (25) was identical for all viral strains, except for a single aa change at aa 362 specified by the avirulent, cell culture-adapted strain EBCV-L9 (Fig. 2). Furthermore, the S1A epitopes of HCV-OC43 and the BCV Mebus strain were identical (21). Monoclonal antibodies (MABS) against the

EBCV Mebus cross-reacted with different animal and human coronaviruses (25). Therefore, it is likely that these antibodies react with the S1A epitope. The hypervariable region of the S glycoprotein contains the S1B immunoreactive epitope which is the target for virus neutralizing Mabs (25). Four RBCV-specific aa substitutions at aa 510, aa 531, aa 543 and aa 578 were located within or proximal to this epitope. Based on the observed aa changes, it can be predicted that Mabs specific for this region may be able to distinguish between respiratory, enteric and vaccine BCV strains.

The BCV S2 subunit of S induced cell fusion when it was expressed in insect cells, indicating that S2 contained membrane fusion domains (26). The hydrophobic and heptad repeat regions of S2 are believed to form the coiled-coil structure of the oligomeric S protein that have been associated with fusion activity. Specifically, three aa changes within a predicted heptad region of the MHV S2 subunit were shown to be responsible for pH-dependent cell fusion (27). RBCV strains LSU and OK are highly fusogenic in cell culture (data not shown). Additional experimentation is required to assess whether the aa change of Ala 769 to Ser immediately after the proteolytic cleavage site and the aa change of Asp 1026 to Gly within the heptad repeat are responsible for the extensive cell fusion induced by RBCV.

RBCV-specific genetic alterations between the S and E genes. The RBCV genomic regions between the S and E genes contained many nucleotide substitutions, deletions and insertions. HCV-OC43 and three porcine HEV strains specified deletions within the 4.9 and 4.8 kDa ORFs, indicating that they are not essential for virus replication. Similarly, the high number of mutations within the RBCV 4.8 and 4.9 kDa ORFs suggests that these ORFs are not essential for virus replication in cell culture (Table 1). The 65 nt leader of a cloned BCV defective interfering (DI) RNA when mapped by mutations, could be converted rapidly to the wild-type leader of a helper virus following DI RNA transfection into helper

Fig. 2. Comparison of the predicted amino acid sequences of the BCV S glycoprotein specified by different strains. Amino acids that are different in at least one strain are shown, except aa 1, aa 768 and aa 1363 which are included as reference points. * indicates unique amino acid changes for each strain. Boxed amino acids are common among different strains. Light-gray boxes contain RBCV-specific, dark boxes contain virulent-specific, and clear boxes contain EBCV-specific aa changes. aa 1–17 is the putative signal peptide; aa 351–403 is the S1A immunoreactive domain; aa 517–621 is the S1B immunoreactive domain; aa 452–593 is the hypervariable region; aa 955–992 is the hydrophobic region; aa 993–1032 is the heptad repeat sequence; aa 1312–1325 is the carboxy-terminal anchor sequence.

		1	4.9 kDa	Carboxy-end of Spike		70		
	Mebus	ATGACGACTA	AGTTTCGTCTT	TGATTTTATTG	GCTCCTGACG	ATATATTACA	TCCTTCAAT	CATGTGAAGC
	LY	ATGACGACTA	AGTTTCGTCTT	TGATTTTATTG	GCTCCTGACG	ATATATTACA	TCCTTCAAT	CATGTGAAGC
	LSU	ATGACGACTA	AGTTTCGTCTT	TGATTTTATTG	GCTCCTGACG	ATATATTACA	TCCTTcCAAT	CATGTGAATc
	OK	ATGACGACTA	AGTTTCGTCTT	TGATTTTATTG	GCTCCTGACG	ATATATTACA	TCCTTcCAAT	CATGTGAATc
HCVOC43	peptide 11	ATGACGACTA	AGTTTCGTCTT	TGATTTCATTG	cactgatctc	tTgTtagA..
HEVNT9	peptide 24	ATGACGACTA	AGTTTCGTCTT	TGTTTTACTG	aCTCtTGACc	ATATAgTA..
HEVVW572	peptide 24	ATGACGACTA	AGTTTCGTCTT	TGATTTTAcTG	aCTCtTGACG	ATATAgTA..
HEV67N	peptide 20	ATGACGACTA	AtTTTCGTCTT	TGgTTT....Cc	ATATAgTA..
		71						140
	Mebus	TA...ATTAT	AAGACCCATT	GAGGTCGAGC	ATATTATAAT	AGCTACCACA	ATGCCTGCTG	TtTAGTGGGT
	LY	TAattATTAT	AAGACCCATa	GAGGTCGAGC	ATATTATAAT	AGCTACCACA	ATGCCTGCTG	TtTAGTGGGT
	LSU	TA...ATTAT	AAGACCCATT	tAGGTCGAGC	ATATTATAAT	AGCTACCACA	ATGCCTGCTG	TtTAGTGGGT
	OK	TA...ATTAT	AAGAcTcATT	GAGGTCGAGC	ATATTATAAT	AGCTACCACA	ATGCCTGCTG	TtTAGTGGGT
HCVOC43	peptide 11
HEVNT9	peptide 24
HEVVW572	peptide 24
HEV67N	peptide 20
		141			4.8 kDa			210
	Mebus	ACTGTGTCTT	ATATAACTAG	TAAACCTGTA	ATGCCAATGG	CTACAACCAT	TGACGGTACA	GATTATACTA
	LY	ACTGTGTCTT	ATATAACTAG	TAAACCTGTA	ATGCCAATGG	CTACAACCAT	TGAtGGTACA	GATTATACTA
	LSU	ACTGTGTCTT	ATATAACTAG	TAAACCTGcA	ATGCCAATGG	CTACAACCAT	TGAaGGTgCA	GATTATACTA
	OK	ACTGTGTCTT	ATATAACTAG	TAAACCTGTA	ATGCCAATGG	CTACAACCAT	TGAtGGTACA	GATTATACTA
HCVOC43	peptide 11
HEVNT9	peptide 24
HEVVW572	peptide 24
HEV67N	peptide 20
		211						280
	Mebus	ATATTATGCC	TAGTACTGTT	TCTACAACAG	TTTATTTIAGG	CTGTTCTATA	GGTATTGACA	CTAGCACCAC
	LY	AcATTATGCC	TAGTACTGTT	TCTACAACAG	TTTATTTIAGG	CgGTTCTATA	GGTATTGATa	CTAGCACCAC
	LSU	AcATTATGCC	TAtTACTGTT	ctTACAACAG	TTTATTTIAGG	CgtTTCTATA	GGTATTGACA	CTAGCACCAC
	OK	ATATTATGCC	TAtTACTGTT	TtTACAACAG	TTTATTTIAGG	CgtTTtTATA	GGTATTGACA	CTAGCACCAC
HCVOC43	peptide 11
HEVNT9	peptide 24
HEVVW572	peptide 24
HEV67N	peptide 20
		281						350
	Mebus	TGGTTTTACC	TGTTTTTCAC	GGTACTAGTT	CCAAACCATA	TTATAAITTa	GGTAGACCTT	ATAACTTTAA
	LY	TGGTTTTACC	TGTTTTTCat	GGTACTAGTT	CCAAACCATA	TTATAAITcA	GGTAGACCTT	ATAACTTTAA
	LSU	TGGTTTTACC	TGTTTTTCAC	GGTACTAGTT	CCAAACCATA	TTATAAITTa	GGTAGACCTT	ATAACTTTAA
	OK	TGGTTTTACC	TGTTTTTCAC	GGTACTAGTT	CCAAACCATA	TTATAAITcA	GGTAGACCTT	ATAACTTTAA
HCVOC43	peptide 11tCtTT	tTgcaaTctA
HEVNT9	peptide 24ActIT	A.....A
HEVVW572	peptide 24ActIT	A.....A
HEV67N	peptide 20ActIT	A.....A
		351				394	12.7 kDa	
	Mebus	GCATTATTAA	TGCCCAAAGT	TTCTAAGGTC	ACGCCCTAGT	AATG		
	LY	GCATTATTAA	TGCCCAAAGT	TcCTAAGGcC	ACGCCCTAGT	AATG		
	LSU	GCATTAT...	TGCCCAAAGT	TTtTAAGGcC	ACGCCCTAGT	AATG		
	OK	GCATTAT...	TGCCCAAAGT	TTtTAAGGcC	ACGCCCTAGT	AATG		
HCVOC43	peptide 11	GCATT.....	TGtTAAAGT	TctTAAGGcC	ACGCCCTAtT	AATG		
HEVNT9	peptide 24	GCATT.....	TGCCCAAAGT	TTtTAAAGGct	ACGCCCTAtT	AATG		
HEVVW572	peptide 24	GCATT.....	TGCCCAAAGT	TTtTAAAGGcC	ActCCCTAgT	AATG		
HEV67N	peptide 20	GCATT.....	TGCCCAAAGT	TTtTAAAGGct	ACGctCTAtT	AATG		

Fig. 3. Alignment of BCV nucleotide sequences spanning the region demarcated by the 3' end of the S gene and the 5' end of the 12.7 kDa ORF. Alignment was performed using the multiple sequence alignment with the hierarchical clustering software MultAlin (33). The leader-mRNA junction sequences are underlined. Start and stop codons for each ORF are represented by bolded letters. Nucleotide deletions are noted as dotted lines. Nucleotide differences among strains are noted as small letters, while conserved nucleotides are capital letters.

amino acids		1	21	89	137	186	192	252	253	262	274	279
									S* S* Y* F* R* K* S* C* Q*			
G	RBCV LSU-94LSS-051-2	ATG	Q*	E*	N	A*	I*	K		N	S*	TAA
									S* S* Y* F* R* K* S* C* Q*			
G	RBCV OK-0514-3	ATG	Q*	E*	N	A*	I*	K		N	S*	TAA
									S* F* Y* F* R* K* S* C* Q*			
G	EBCV LY138	ATG	E	D	D*	V	I*	K		N	S*	TAA
									V L I S V S L V			
G	EBCV Quebec	ATG	E	D	N	V	S	K		N	P	TAA
	amino acids	1	21	89	137	186	192	252	253	261	273	278

Fig. 4. Comparison of the predicted amino acid sequences of the 32kDa ORF specified by different BCVs. Amino acids which are different in each BCV strain in comparison to BCV-Quebec are shown. * represent amino acid differences from BCV-Quebec. Amino acids in each BCV strain which are different from the BCV-Quebec as the result of the double-frame shift mutations are represented by bolded letters.

virus-infected cells (28). Nucleotide substitutions mapped the crossover region to a 24-nucleotide segment that starts from the last nt of the leader-mRNA junction sequence and extends further downstream. The RBCV isolates LSU, OK, as well as RBCV AZ-26649-2 (AZ) and TX-671-2 (TX) isolated from California and Texas cattle, respectively (data not shown), contained a four nucleotide deletion located within this 24 nucleotide segment (Fig. 3). This deletion may alter the recombination frequency between the leader and the leader-mRNA junction sequence immediately upstream of the 12.7kDa subgenomic mRNA, and cause either inhibition or enhancement of the putative 12.7kDa transcription and subsequent protein expression.

Genetic differences between virulent and avirulent BCV strains. The S glycoprotein contained 7 aa substitutions which were common for all virulent strains (aa 33, 40, 248, 470, 965, 1241 and 1341). Three mutations within the SI portion of S caused conservative aa changes, while one non-conservative aa change of His 470 to Asp was located within the S1 hypervariable region. All three mutations within S2 caused non-conservative amino acid changes. Amino changes within S1 and S2 may affect the structure and function of the S glycoprotein and alter the pathogenetic potential of these viruses. The 32kDa ORF specified by RBCV virulent strains LSU and OK as well as EBCV LY-138 contained two frame-shift mutations which resulted in a 9 aa segment near the carboxy-terminus which was different from the corresponding amino acid sequence specified by the avirulent EBCV-Quebec strain (Fig. 4). A similar double frame shift mutation was found in the 32kDa ORF specified by HCV-OC43 (29). These frame shift mutations increased substantially the hydrophilicity of the carboxy terminal portion of the 32kDa protein in virulent versus avirulent strains (data not shown). Sequencing of the corresponding region of the 32kDa protein of the avirulent EBCV L9 and Mebus strains as well as the 32kDa of other virulent strains will substantiate these differences between virulent and avirulent strains. The aa substitution of Gly 53 to Val in the E protein was conserved for all BCV virulent strains (F15, LY-138, OK, LSU), HCV-OC43 (30), and three different porcine HEV strains (22). This mutation may affect the ability of these viruses to invade different tissues, because E is part of the virion and is expressed at infected cell-surfaces (31,32).

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