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The Hemagglutinin/Esterase Glycoprotein of Bovine Coronaviruses: Sequence and Functional Comparisons between Virulent and Avirulent Strains

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The entire nucleotide sequences of the hemagglutinin/esterase (HE) genes specified by the highly virulent strain LY138 and the avirulent strain L9 of bovine coronavirus (BCV) were determined. These sequences were compared with recently published sequences of the HE genes of the Quebec and Mebus strains. A large open reading frame of 1272 nt encoding a protein of 424 amino acid residues was predicted. The putative esterase active site was conserved in the virulent and avirulent BCV strains, indicating that this domain is probably not a determinant for BCV virulence. Four amino acid substitutions occurred between the HE proteins of BCV-L9 and BCV-LY138 (Leu to Pro at 5, Leu to Val at 103, Ser to Pro at 367, and Thr to Asn at 379). Monoclonal antibodies specific for the HE glycoprotein inhibited the hemagglutination and acetylerase activities of BCV-L9, but showed no inhibitory effect on the acetylerase activity of BCV-LY138. These results suggest that at least one epitope is located proximal to one of the three strain-specific amino acids. Four S-specific monoclonal antibodies inhibited hemagglutination but not acetylerase activity of BCV-L9, implying that the S glycoprotein can promote hemagglutination of chicken erythrocytes in addition to the HE glycoprotein. © 1991 Academic Press, Inc.

Bovine coronavirus (BCV) is a member of the Coronaviridae, possessing a single-stranded, nonsegmented RNA genome of positive polarity (1). The virion contains four major structural proteins: the nucleocapsid protein (N), the transmembrane glycoprotein (M), the spike glycoprotein (S), and the hemagglutinin/esterase glycoprotein (HE) (2). BCV binds to receptors on erythrocytes and possesses a receptor-destroying enzyme similar to the HEF glycoprotein of influenza C virus (3-12). The receptor-destroying activity of the HEF protein is associated with the acetylerase (AE), which hydrolyzes an ester linkage to release the acetyl group from position C-9 of *N*-acetyl-9-*O*-acetylneuraminic acid (3-5, 7). The 9-*O*-acetyl residue is crucial for influenza C virus recognition of the glycoprotein receptor, a major determinant for viral attachment to cells and initiation of viral infection (13). By analogy to influenza C virus, the BCV HE is probably involved in virus attachment and entry during the infectious process in addition to the S glycoprotein. Recently, the BCV HE protein was shown to induce neutralizing antibodies both *in vitro* and *in vivo* (14, 15). Four neutralizing epitopes were found on the HE protein of the BCV-Quebec strain, however, their locations and functional properties such as receptor-binding and receptor-destroying activities were not defined (15). The HE protein is N-glycosylated with a molecular weight of 62 to 65

kDa in its reduced form and exists as a disulfide-linked homodimer of 124 to 140 kDa in its unreduced form (15-18). Interestingly, this protein is present only on some coronaviruses, such as human coronavirus (HCV)-OC43, hemagglutinating encephalomyelitis virus of swine, and turkey enteric coronavirus (19), while others, such as avian infectious bronchitis virus (IBV), porcine transmissible gastroenteritis virus, and feline infectious peritonitis virus lack this protein (1). The genome of mouse hepatitis virus (MHV) A59 contains an open reading frame (ORF) coding for an HE protein. However, this protein is not expressed in infected cells (20, 21). The HE protein of MHV-JHM was shown to have AE activity (22). It was assumed that the HE gene is not required for viral infectivity in MVH-A59 and MHV-JHM (21). Clearly, the role of the HE glycoprotein in coronavirus evolution, replication, and pathogenesis remains enigmatic.

To study the role of the HE glycoprotein in BCV infection we sequenced and cloned the HE genes of one virulent and one avirulent BCV strain. We report here the complete nucleotide and predicted amino acid sequences of their HE genes and their comparison with recently published HE gene sequences of the Quebec and Mebus strains. We also investigated the biological functions and antigenicity of the HE glycoprotein between the virulent BCV-LY138 and the avirulent BCV-L9 strains using monoclonal antibodies (MAbs).

Strain BCV-L9 was derived from BCV-Mebus and passaged through different nonpolarized and highly polarized cells over 80 passages (23). The highly viru-

Sequence data from this article have been deposited with the EMBL/Genbank Libraries under Accession Nos. M76372 for BCV-L9 and M76374 for BCV-LY138.

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lent wild-type strain BCV-LY138 was isolated from diarrheal fluid of a diseased calf in 1965 in Utah (24) and maintained in calves through oral inoculation since then (25). This strain replicates only in human rectal tumor (HRT-18) cells but not in numerous bovine cells (23). BCVs were propagated in HRT-18 cells and purified as described previously (23, 26).

Hemagglutination (HA) and hemagglutination inhibition (HI) tests were performed according to Herrler *et al.* (4) and Zhang *et al.* (27) employing chicken and mouse erythrocytes. Five MAbs specific for gp100/S and one MAb specific for N protein of strain BCV-L9 were characterized previously (28). Three MAbs specific for the HE glycoprotein of BCV strain S2 were obtained from Dr. Snodgrass (Moredun Research Institute, Edingburgh, Scotland). These MAbs were used in HI and acetylesterase inhibition (AEI) assays.

The AE activity was determined according to Herrler *et al.* (3) and Vlasak *et al.* (9). Briefly, 10 μ l of purified viral preparation was incubated with 200 μ l bovine submaxillary mucin (BSM type I, Sigma; 25 mg/ml in PBS) at 37°. At different time points, the mixture was assayed for free acetate with a commercial test kit (Boehringer-Mannheim, FRG). A sample of BSM incubated with the same amount of purified viruses at 4° served as a control. The value of the control samples was subtracted from that of the samples incubated at 37°. Inhibition of AE activity was performed as described previously (6). Briefly, 10 μ l of purified virus preparation was incubated with 10 μ l of undiluted MAbs for 1 hr at room temperature. Following the addition of 5 mg of BSM, the incubation temperature was raised to 37°, and 30 min later the amount of acetate released from BSM was determined as described above. The values were compared with those of the control sample incubated in the absence of antibodies.

Viral RNA was isolated from infected HRT-18 cells using isothiocyanate/cesium chloride gradients as described previously (26, 29). As control, RNA was isolated from uninfected cells. cDNA synthesis, PCR amplification, and DNA sequencing were performed as reported recently (26). Nucleotide sequences were determined in both directions at least once.

We sequenced the complete HE genes of the virulent BCV-LY138 and the avirulent BCV-L9 strains. The alignments of nucleotide and deduced amino acid sequences are presented in Figs. 1 and 2, respectively. These sequences were also compared with recently published sequences for the Quebec (30) and Mebus strains (31). All of the HE genes contained a large ORF of 1272 nt encoding a predicted protein of 424 amino acid residues, which has a calculated molecular weight of approximately 42.5 kDa. The only variations among these sequences consisted of nucleotide sub-

stitutions. Frameshift, deletion or insertion, and nonsense mutation were not observed. There were 15 nt substitutions between the virulent and the avirulent strains. Eleven of them occurred in the third codon position and did not result in amino acid changes. The HE gene sequence of the Quebec strain differed by only 1 base from that of BCV-L9 (A to T transition at nt 522), which did not cause an amino acid change. In the Mebus strain, only 1 base differed from the sequence of BCV-L9 (G to C at nt 322), resulting in a Leu to Val change at aa 103. There were several features conserved in all BCV strains: the consensus intergenic sequence CTAAAC upstream of the HE gene; two non-overlapping internal ORFs of 408 (ORF1) and 249 (ORF2) bases; a hydrophobic putative signal sequence of 18 amino acids at the N-terminus; an extremely hydrophobic region of 26 amino acids near the C-terminus which may serve as a potential membrane-anchoring domain; a stretch of 10 hydrophilic amino acids at the C-terminus which may be the intravirion domain. The 9 potential N-linked glycosylation sites, the 14 cysteine residues, and the putative active site for neuraminatase-O-acetylesterase activity, F-G-D-S, at the N-terminal of the HE peptide were conserved in all BCV strains.

To examine whether there are differences between HA and AE activities specified by the two BCV strains, purified virions were employed in HA and AE assays. As shown in Table 1, both strains exhibited AE activity as measured by their ability to release acetate from the substrate BSM, when a similar amount of purified virions was used. Strain BCV-LY138 exhibited much lower HA activity with chicken erythrocytes than with mouse erythrocytes in comparison with BCV-L9, indicating that the two BCV strains may have a different binding avidity for receptors on chicken and mouse erythrocytes.

MAbs directed against HE, S, and N proteins were analyzed for their ability to inhibit HA and/or AE activities. As shown in Table 2, three MAbs specific for the HE glycoprotein effectively inhibited the HA activity of BCV-L9 (HI titers ranged from 512 to 8192), whereas the HA activity of BCV-LY138 was only slightly inhibited by these MAbs (HI titer was 16). Likewise, the AE activity of BCV-L9 was greatly inhibited by the HE-specific MAbs (reduction of released acetate in the range of 49 to 95%), while that of BCV-LY138 remained unaffected. MAb 46 specific for the N protein had neither of the HI and AEI functions.

Four MAbs specific for gp100/S of BCV-L9 showed different reactivities with BCV-L9 and BCV-LY138 in Western blotting and neutralization assays as reported previously (28). MAbs 43C2, 34B8, and 38 inhibited the HA activity of BCV-L9 at titers ranging from 128 to

BCV-L9	<u>CTAAACTCAG</u> TGAAAATGTT TTTGCTTCTT AGATTTGTTT TAGTTAGCTG CATAATGGT AGCCTAGGTT TTGATAACCC TCCTACCAAT GTGTTCGC	100
BCV-LY	*** C	
BCV-L9	ATTTAAATGG AGATTGGTTT TTATTGGTG ACAGTCGTTT AGATTGTAAT CATGTTGTTA ATACCAACCC CCGTAATTAT TCTTATATGG ACCTTAATCC	200
BCV-LY		
BCV-L9	TGCCCTGTGT GATTCGTGTA AAATATCATC TAAAGCTGGC AACTCCATTT TTAGGAGTTT TCACCTTACC GATTTTTATA ATTACACAGG CGAAGGTCAA	300
BCV-LY		
BCV-L9	CAAATTATTT TTTATGAGGG TCTTAATTTT ACGCCTTATC ATGCCTTTAA ATGCACCACT TCTGGTAGTA ATGATATTTG GATGCAGAAT AAAGGCTTGT	400
BCV-LY	G	
BCV-L9	TTTACACTCA GGTTTATAAG AATATGGCTG TGTATCGCAG CCTACTTTT GITTAATGTAC CATATGTTA TAATGGCTCT GCACAATCTA CAGCTCTTTG	500
BCV-LY		
BCV-L9	TAAATCTGGT AGTTTAGTTC TTAATAACCC TGCATATATA GCTCGTGAAG CTAATTTTGG GGATTATTAT TATAAGGTTG AAGCTGACTT TTATTGTCA	600
BCV-LY	T	
BCV-L9	GGTTGTGACG AGTATATCGT ACCACTTTGT ATTTTAAACG GCAAGTTTTT GTCGAATACA AAGTATTATG ATGATAGTCA ATATTATTTT AATAAGACA	700
BCV-LY		
BCV-L9	CTGGTGTAT TTATGGTCTC AATTCTACTG AAACCAATAC CACTGGTTTT GATTTTAAIT GTCATTATTT AGTTTTACCC TCTGGTAATT ATTTAGCCAT	800
BCV-LY		
BCV-L9	TTCAAATGAG CTATTGTAA CTGTTCTTAC GAAAGCAATC TGTCTTAAACA AGCGTAAGGA TTTTACGCCT GTACAGGTTG TTGATTCACG GTGGAACAAT	900
BCV-LY	T C T	
BCV-L9	GCCAGGCAGT CTGATAACAT GACGGCGGTT GCTTGTCAAC CCCCCTACTG TTATTTTCGT AATTCTACTA CCAACTATGT TGGTGTTTAT GATATCAATC	1000
BCV-LY		
BCV-L9	ATGGGGATGC TGGTTTTACT AGCATACTCA GTGGTTTGTT ATATGATTC ACTTGTTTTT CGCAGCAAGG TGTTTTTAGG TATGATAATG TTAGCAGTGT	1100
BCV-LY	C	
BCV-L9	CTGGCCTCTC TATTCTATG GCAGATGCC TACTGCTGCT GATATTAATA CCCCTGATGT ACCTATTGT GTGTATGATC CGCTACCACT TATTTTGCTT	1200
BCV-LY	CC T T A	
BCV-L9	GGCATCCTTT TGGGTGTGTC GGTCAATAAT ATTGTAGTTT TGTGTTATA TTTTATGGTG GATAATGGTA CTAGGCTGCA TGATGCTTAG ACCATAATCT	1300
BCV-LY	T C ***	
BCV-L9	<u>AAAC</u>	
BCV-LY		

Fig. 1. Nucleotide sequence comparison of the HE genes of the virulent strain BCV-LY138 and the avirulent strain BCV-L9. cDNA synthesis and PCR amplification were performed as described previously (26). Briefly, the first-strand cDNA was synthesized by reverse transcription using an antisense primer 3'S95 (5'-ATGGAAACCGTAGTAGTACACTT-3', representing the sequence at position 75-95 of the S genes) according to the published sequence (26). The reactions for the second-strand cDNA synthesis and double-stranded cDNA amplifications were carried out in PCR using the 3'S95 and a sense primer 5'HE (5'-GGTTTTATGAATCTCCAGTTGAA-3', corresponding to the sequence upstream of the HE gene) according to the published sequence (30). Two additional internal primers (5'521: 5'-GGCTCTGCACAATCTACAGC-3'; 3'568: 5'-TAGAAC-TAAAACCTACCAGATT-3') were used for BCV-LY138 in order to generate two overlapping fragments for DNA sequencing. DNA sequencing was carried out with the modified dideoxynucleotide chain termination procedure (38) using Sequenase (USB, Cleveland, OH). 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). The consensus sequences CTAAAT are underlined, and the start and stop codes are marked by asterisks.

4096, whereas they failed to inhibit the HA activity of BCV-LY138. Interestingly, the MA b 44, which was reactive only with BCV-L9, but not BCV-LY138 in Western blots (28), inhibited both strains at very high titers (4096 HI units). Pretreatment of these MAbs with kaolin, potassium periodate, and heat did not eliminate their HI function, but caused the HI titers to decrease by twofold. These S-specific MAbs failed to inhibit the AE activity of both strains. Antiserum 1745 inhibited HA and AE functions of both strains (Table 2).

Sequence comparisons reveal that the HE genes specified by the virulent strain BCV-LY138 and the avirulent strain BCV-L9 were highly conserved despite their different origins (homology: nt = 98.8%, aa = 99.1%). The predicted amino acid sequences of

BCV HE genes are 60 and 28% identical with the corresponding deduced amino acid sequences of MHV-JHM mRNA 2b (21) and the HEF (HA1) of influenza C virus (32), respectively, suggesting a common ancestral origin. The HE genes of a bovine respiratory coronavirus strain and HCV-OC43 are also highly conserved (Zhang *et al.*, submitted for publication). Interestingly, a proline substitution occurred in the signal peptide between the avirulent BCV-L9 and the virulent BCV-LY138 strains (at aa 5). Whether this substitution could alter the maturation and intracellular transport of the HE glycoprotein remains to be elucidated. Three additional amino acid substitutions occurred between the HE proteins of BCV-L9 and BCV-LY138: Leu to Val (at aa 103), Ser to Pro (at aa 367), and Thr to Asn (at aa

BCV-L9	<u>MPILLRFLV</u> <u>SCIIGSLGFD</u> <u>NPPTNVVSHL</u> <u>NGDWFLPGDS</u> <u>RSDCNHVVNT</u> <u>NPRNYSYMDL</u> <u>NPALCDSGKI</u> <u>SSKAGNSIFR</u> <u>SFHPTDFYNY</u> <u>TGEGQQIIFY</u>	100
BCV-LY	<u>P</u> <u>▲</u> <u>****</u> <u>=</u> <u>=</u>	
BCV-L9	<u>EGLNFTPIYA</u> <u>FKCTTSGSND</u> <u>IWMQNKGLFY</u> <u>TQVIKNMAVY</u> <u>RSLTFVNVPI</u> <u>VYNGSAQSTA</u> <u>LCKSGSLVLN</u> <u>NPAYIAREAN</u> <u>FGDYIYKVEA</u> <u>DFYLSGCDEY</u>	200
BCV-LY	<u>V=</u> <u>=</u>	
BCV-L9	<u>IVPLCIFNGK</u> <u>FLSNTRYDD</u> <u>SQYFKNKDTG</u> <u>VIYGLNSTET</u> <u>ITTGFDNFCH</u> <u>YLVLPNGNYL</u> <u>AINSELLTV</u> <u>PTKAICLNKR</u> <u>KDFTPVQVVD</u> <u>SRWNNARQSD</u>	300
BCV-LY	<u>=</u>	
BCV-L9	<u>NMTAVACQPP</u> <u>YCYFRNSTTN</u> <u>YVGVYDINHG</u> <u>DAGFTSILSG</u> <u>LLYDSPCFSQ</u> <u>QGVFRYDNVS</u> <u>SVWPLYSYGR</u> <u>CPTAADINTP</u> <u>DVPICVYDPL</u> <u>PLILLGILLG</u>	400
BCV-LY	<u>=</u> <u>=</u> <u>=</u> <u>=</u> <u>=</u> <u>P</u> <u>N</u>	
BCV-L9	<u>VAVIIIVLL</u> <u>LYFMVDNGTR</u> <u>LHDA</u> 424	
BCV-LY	<u>=</u>	

Fig. 2. Amino acid sequence comparisons of the predicted HE proteins of the virulent BCV-LY138 and the avirulent BCV-L9 strains. The predicted signal peptide and intramembrane-anchoring sequences are underlined. The arrowhead indicates the predicted cleavage site of the signal peptide. The nine predicted N-linked glycosylation sites are double-underlined. The putative esterase active site (FGDS) is marked by asterisks. Fourteen cysteine residues are indicated by dots. Only different amino acids are shown in the second amino acid sequence.

379) (see Fig. 2). MAbs specific for the HE glycoprotein reacted differently with the two strains in HI and AEI assays (Table 2). The epitopes on the HE proteins seemed to be continuous epitopes since these MAbs reacted with the HE in Westerns under denatured conditions (data not shown). These results imply that at least one epitope is located proximal to one of the three strain-specific amino acids.

Importantly, the sequences of the putative esterase active domain were conserved in all virulent and avirulent BCV strains, MHV-A59, MHV-JHM, and in the influenza C virus sequenced so far. This indicates that the esterase active site is essential for the structure and function of the HE glycoprotein. However, this domain is probably not a determinant for BCV virulence, since it is conserved in both virulent and avirulent strains. All three MAbs against HE inhibited both HA and AE activities of BCV-L9. This implies that these two activities are functionally linked to each other, however, we can not exclude the possibility that the two functional domains may be located at different regions of the HE molecule. It is worth noting that in the case of influenza

C virus some MAbs inhibited either HA or AE activity, but not both activities (5).

BCV binds to receptors on erythrocytes facilitating hemagglutination. This activity was reported earlier to be associated with the HE glycoprotein (14, 16, 30, 33). Since the S glycoprotein of coronaviruses is involved in virus attachment to permissive cells, we reexamined the possibility that the S glycoprotein may also recognize receptors on erythrocytes to facilitate hemagglutination. As shown in Table 2, the results confirm our previous observation that the S glycoprotein of BCV-L9 is involved in agglutination of chicken erythrocytes and that this activity can be blocked by S-specific MAbs (34). These results are also in agreement with the observation that the isolated S glycoprotein of

TABLE 1

HEMAGGLUTINATION AND ACETYLESTERASE ACTIVITIES OF THE VIRULENT STRAIN BCV-LY138 AND THE AVIRULENT STRAIN BCV-L9

BCV strain	HA (titer)		AE ($\mu\text{g acetate}/\mu\text{l}$) ^a
	Chicken RBC	Mouse RBC	
L9 (purified virus)	1024	8192	2.148
LY138 (purified virus)	256	16,384	2.860

^a Acetylcsterase activity was determined by releasing acetate from BSM.

TABLE 2

INHIBITION OF THE HEMAGGLUTINATION AND ACETYLESTERASE ACTIVITIES OF BOVINE CORONAVIRUSES BY MONOCLONAL ANTIBODIES

MAb		Inhibition of			
		Hemagglutination (HI titer)		Acetylcsterase (%)	
Identif.	Specificity	L9	LY138	L9	LY138
44	Anti-S	4096	4096	<10	<10
43C2	Anti-S	128	<4	<10	<10
38	Anti-S	4096	<4	<10	<10
31	Anti-S	<4	<4	<10	<10
34B8	Anti-S	128	<4	<10	<10
46	Anti-N	<4	<4	<10	<10
S2/1	Anti-HE	512	16	95	<10
S2/4	Anti-HE	4096	16	49	<10
S2/7	Anti-He	8192	16	90	<10
Antiserum					
1745	BCV	128	64	52	40

BCV-L9 was able to agglutinate chicken erythrocytes (35). In contrast, there is a discrepancy between our results and the observation by Parker *et al.* (30) that MAbs specific for the S did not inhibit HA activity of BCV-Quebec. One possible explanation is that there is a strain difference in receptor-binding activities of S glycoproteins. This is supported by the observed differences in agglutination patterns of different BCV strains with chicken erythrocytes (Table 1; Storz *et al.* unpublished observation). It is also possible that the mouse ascites used as the MAb source contained non-specific inhibitors which could also inhibit hemagglutination. This is unlikely, however, because pretreatment of the ascites with kaolin, heat, and potassium periodate did not eliminate the HI activity. These treatments were employed to eliminate nonspecific inhibitors in sera derived from humans and animals which may interfere with detection of influenza antibodies (27, 36). The anti-S-specific MAbs did not react with the HE glycoprotein in Western blots (28). This evidence argues against the possibility that the ascites were contaminated with anti-HE MAbs. Consequently, these results suggest that the BCV S glycoprotein is involved in agglutination with chicken erythrocytes (35). This may explain why some IBV strains, which lack a HE glycoprotein, exhibit HA activity (37). The receptor determinants on chicken erythrocytes specific for BCV S protein appear to be similar to those of the HE protein (35). However, we cannot exclude the possibility that anti-S MAbs inhibit HA activity of BCV by steric hindrance of the antibody molecule on HE protein.

The presence of the HE in BCV and other hemagglutinating coronaviruses leads us to hypothesize that the HE glycoprotein may share the receptor-binding function with the S glycoprotein and that these two molecules act synergistically during viral infection. Experiments which utilize cloned S and HE genes to express and to characterize their functional properties, including their binding ligands on host cells, will provide further insight into BCV molecular pathogenesis.

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