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Original Article

Cross-reactivity of Vaccine and Fields Strains of Bovine Coronaviruses in Korea

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Bovine coronavirus (BCoV) causes severe diarrhea in newborn calves, and is associated with winter dysentery in adult cattle and respiratory infections in calves and feedlot cattle. Although the Korean BCoV vaccine strain, BC94, was isolated in 1995, there has still been no report of a molecular characterization of the vaccine strain. To characterize the vaccine strain, relationships between BC94 and field strains were investigated, based on sequence analysis and cross-immunity. We determined the complete sequences of the HE, N, and S genes from BC94 and four NVRQS isolates (SUN5, A3, 0501, 0502). Due to its major role in antigenicity, the spike proteins of the BCoVs were analyzed. BC94 showed distinctive genetic divergence from field isolates collected from 2002 to 2005. BC94, SUN5, and A3 had no virulence-specific sequence and there was a single amino acid change, from asparagine to lysine at residue 175, in the polymorphic region. Strains 0501 and 0502 had virulence-specific sequences at all seven sites. Although the recently isolated Korean BCoVs and BC94 were genetically different, the cleavage site of spike genes at 763~768 (KRRSRR) and the antigenic domain II of the spike protein, amino acid position 528, were conserved in all NVRQS isolates. The antigenic relatedness of KCD9, representative of recent Korean BCoVs, was compared with the Korean vaccine strain BC94. KCD9 showed cross-reactivity against BC94 by virus neutralization (VN) test. These results suggest that BC94 is antigenically closely related to field isolates and is still effective as a vaccine strain.

Key Words: Bovine coronavirus; Vaccine strain; Cross-reactivity

INTRODUCTION

Bovine coronavirus (BCoV) is a member of the family *Coronaviridae* of the order *Nidovirales* (1) and causes severe diarrhea in neonatal calves (CD), winter dysentery (WD) in adult cattle, and respiratory disease in feedlot cattle (2~6). BCoV is a 30-kb single-stranded, plus-sense RNA genome virus, consisting of five major structural proteins: nucleocapsid (N) protein, the transmembrane (M) protein, the small envelope (E) protein, the spike (S) protein, and the hemagglutinin-esterase (HE) protein (7). Both S and HE glycoproteins are able to induce erythrocyte hemagglutination, by binding to the *N*-acetyl-9-*O*-acetylneuraminic acid (Neu $5,9Ac_2$)-containing receptor, but S protein has been proposed to play the major role to attachment of virus to cell surface receptor (8, 9).

The coronavirus spike (S) protein, which is cleaved at amino acid positions 768 and 769, to form two subunits, S1 (N-terminus) and S2 (C-terminus), has several important functions, including viral attachment to the host cell, mediation of membrane fusion, and antibody neutralization

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(10~17). Chouljenko reported that the spike gene includes a putative signal peptide (aa 1~17), S1A immunoreactive domain (aa 351~403), S1B immunoreactive domain (aa 517~621), hydrophobic region (aa 955~992), heptad repeat sequence (aa 993~1032), and carboxyl-terminal anchor sequence (aa 1312~1325) (18).

The HE and S glycoproteins are more immunogenic than the N protein and M glycoprotein (19). The S2 subunit is conserved, but a single amino acid change (528V) in the S1 subunit confers resistance to the viral neutralization (20, 21).

Molecular analyses of American and Canadian isolates have been conducted by comparing their S and HE genes (22-27). Korean calf diarrhea (KCD) strains and Korean winter dysentery (KWD) strains were isolated during the period from 2002 to 2004 and comparative studies have been performed based on their S and HE gene sequences (28~30).

In the present study, we compared recently isolated Korean BCoV and the Korean vaccine strain, based on their immunological and genomic properties to determine whether the recently isolated strains should be considered as new vaccine strains. Cross-reactivity was observed between the Korean vaccine strain and presently widespread bovine coronaviruses.

MATERIALS AND METHODS

Vaccine and field strains

Field strains of BCoV were isolated in Korea from 1994 to 2005 from fecal samples, prepared as described previously (31). Each sample was inoculated in cultures of human rectal tumor cells (HRT-18) and MDBK cells to isolate the virus. The Korean vaccine strain BC94 was isolated in 1994 by the National Veterinary Research and Quarantine Service (NVRQS).

RNA extraction, cDNA synthesis, and RT-PCR

Total RNA was extracted from a 200 μl starting volume of supernatant fluids from infected HRT-18 cells using the

Table 1. The oligonucleotide primers of HE, N and S gene. Mebus strain (GenBank accession No. U00735) used for DNA cloning and sequencing

Gene name	Primer name	Sequence	Location	Reference
S	S1F	5'-ATGTTTTTGATACTTTTAATTTCC-3'	S gene 1~920	30
	S1R	5'-ACACCAGTAGATGGTGCTAT-3'		
	S2F	5'-GGGTTACACCTCTCACTTCT-3'	S gene 782~1550	
	S2R	5'-GCAGGACAAGTGCCTATACC-3'		
	S3F	5'-CTGTCCGTGTAAATTGGATG-3'	S gene 1459~2286	
	S3R	5'-TGTAGAGTAATCCACACGT-3'		
	S4F	5'-TTCACGACAGCTGCAACCTA-3'	S gene 2151~3022	
	S4R	5'-CCATGGTAACACCAATCCCA-3'		
	S5F	5'-CCCTGTATTAGGTTGTTTAG-3'	S gene 2691~3606	
	S5R	5'-ACCACTACCAGTGAACATCC-3'		
	S6F	5'-GTGCAGAATGCTCCATATGGT-3'	S gene 3439~4092	
	S6R	5'-TTAGTCGTCATGTGATGTTT-3'		
HE	HEF	5'-GGATCCATGTTTTTGCTTCCT-3'	HE gene 1~1275	
	HER	5'-CTCGAGTTATCGTAGTACGTCGGA-3'		
N	NF	5'-GGATCCATGTTTTTGCTTCCT-3'	N gene 1~1347	
	NR	5'-CTCGAGTTATATTTCTGAGTGTCTTCT-3'		

Table 2. The GenBank accession numbers of reference strains of BCV vaccine and field isolates

Strain	Year	HE	S	N
BC94 Korean vaccine	1994	EU401979	EU401989	EU401985
SUN5	1994	EU401978	EU401988	EU401984
A3	1994	EU401977	EU401987	EU401983
0501	2005	EU401975	EU686689	EU401980
0502	2005	EU401976	EU401986	EU401981
Mebus	1972	U00735	U00735	U00735
L9	1991	M76372	M64667	
Norden vaccine	1991		M64668	
F15	1979		D00731	M36656
LY138	1965	AF058942	AF058942	AF058942
ENT	1998	AF391542	AF391542	AF391542
LSU	1994	AF058943	AF058943	AF058943
OK	1996	AF058944	AF058944	AF058944
KCD1	2004	DQ389642	DQ389632	
KCD2	2004	DQ389643	DQ389633	
KCD3	2004	DQ389644	DQ389634	
KCD4	2004	DQ389645	DQ389635	
KCD5	2004	DQ389646	DQ389636	
KCD6	2004	DQ389647	DQ389637	
KCD7	2004	DQ389648	DQ389638	
KCD8	2004	DQ389649	DQ389639	
KCD9	2004	DQ389650	DQ389640	
KWD1	2002	DQ016118	AY935637	
KWD2	2002	DQ016119	AY935638	
KWD3	2002	DQ016120	AY935639	
KWD4	2002	DQ016121	AY935640	
KWD5	2002	DQ016122	AY935641	
KWD6	2002	DQ016123	AY935642	
KWD7	2002	DQ016124	AY935643	
KWD8	2002	DQ016125	AY935644	
KWD9	2002	DQ016126	AY935645	
KWD10	2002	DQ016127	AY935646	
KWD11	2002	DQ994162	DQ389652	
KWD12	2002	DQ994163	DQ389653	
KWD13	2002	DQ994164	DQ389654	
KWD14	2002	DQ994165	DQ389655	
KWD15	2002	DQ994166	DQ389656	
KWD16	2002	DQ994167	DQ389657	
KWD17	2002	DQ994168	DQ389658	
KWD18	2002	DQ994169	DQ389659	
KWD19	2002	DQ994170	DQ389660	

Trizol reagent (Gibco-BRL, Life Tech, Grand Island, NY, USA). Total cDNAs were synthesized from 2 μg of total RNA using Superscript II RNase H^- reverse transcriptase (Gibco-BRL) with an oligo(dT) primer. Each cDNA was amplified by PCR using gene-specific primers (Table 1), designed according to the published sequences of the S, HE, and N genes of the Mebus strain (GenBank accession No. U00735), described by Park *et al* (30).

DNA sequence analysis and data analysis

RT-PCR products were purified using a Geneclean Turbo kit (Bio 101, Inc., La Jolla, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed using an automated DNA sequencer (ABI PRISM 3730xl; Applied Biosystems Inc., Foster City, CA, USA). Amino acid sequences were deduced using the DNAStar EditSeq software. Nucleotide and amino acid sequence alignments and phylogenic tree construction were performed using Clustal W in the DNAStar MegAlign software.

Virus neutralization (VN) test for cross-reactivity

Guinea pigs were inoculated with BcoV isolate KCD9 and BC94 to produce antisera. Serial two-fold dilutions of serum in α-MEM were mixed with the same volume (100 μl) of KCD9 and BC94 suspensions containing 200 $TCID_{50}$/100 μl and incubated at 37℃ for 1 h. HRT-18 cell monolayers grown in 96-well microplates were washed three times with α-MEM and then inoculated with 100 μl of each virus-serum mixture. Serum samples were tested in triplicate wells. The cells were fixed with 80% acetone 5 days after inoculation and assessed by a direct immunofluorescence assay (32). Virus-neutralizing antibody titers

are expressed as the reciprocals of the highest serum dilution that completely neutralized virus replication. Back titration of each virus was performed.

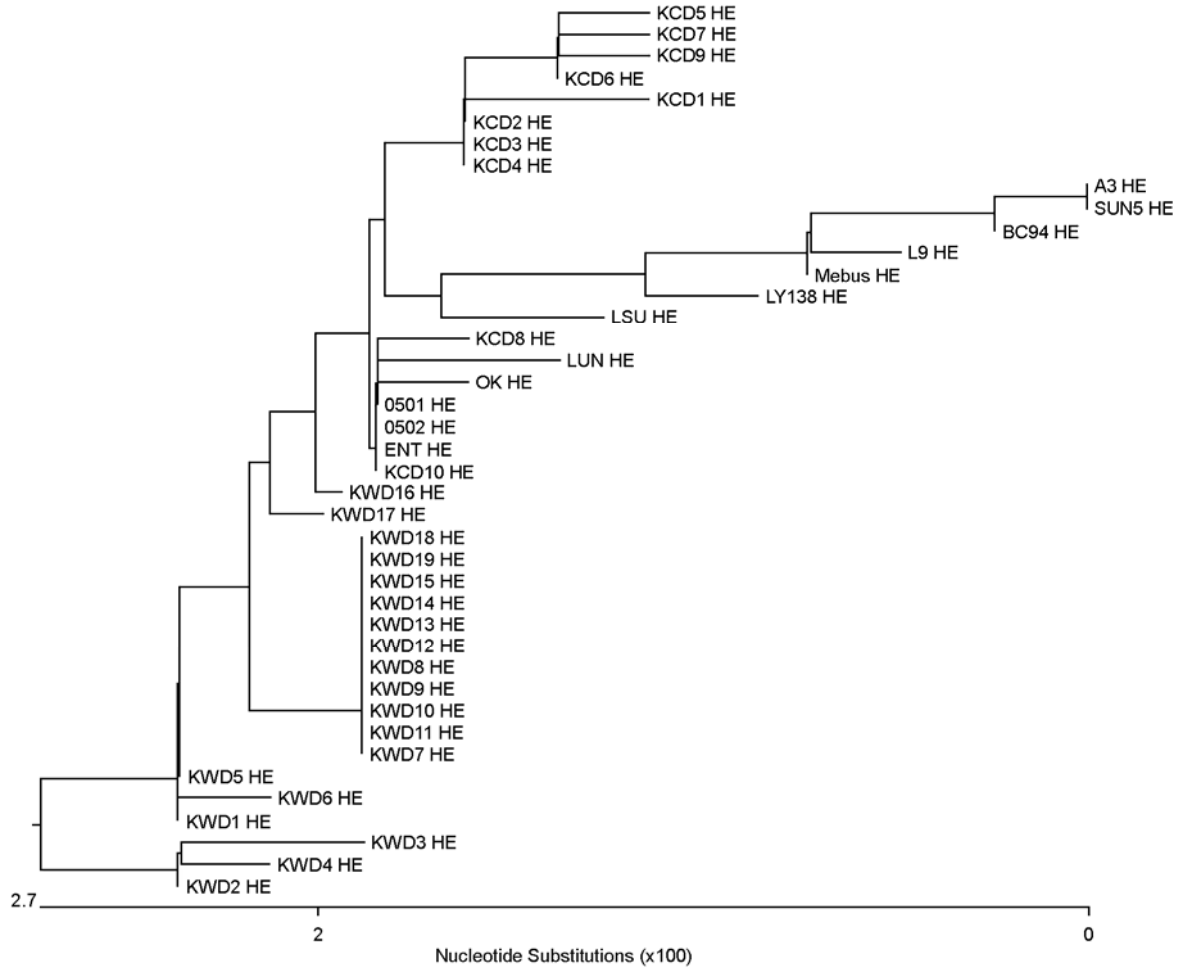

Figure 1. Phylogenic tree of BCV HE. Mebus, L9, F15, LY-138, ENT, vaccine strain M64668, Korean vaccine BC94, SUN5, 0501, 0502, KWDs, and KCDs were analyzed by using Clustal W multiple alignment method of MegAlign program in DNAstar.

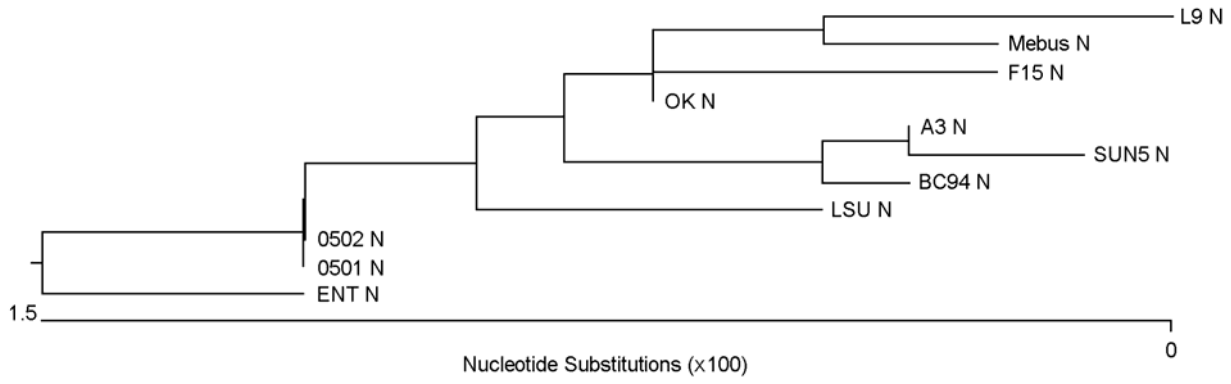

Figure 2. Phylogenic tree of BCV N. Mebus, L9, F15, LY-138, ENT, vaccine strain M64668, Korean vaccine BC94, SUN5, 0501 and 0502 were analyzed by using Clustal W multiple alignment method of MegAlign program in DNAstar.

RESULTS

Molecular analysis of HE gene

The HE genes of BC94 (vaccine strain) and four NVRQS strains (SUN5, A3, 0501, and 0502) each contained an ORF of 1275 nucleotides encoding a predicted product 425 aa residues in length (Table 2). Pair-wise comparisons showed nucleotide and amino acid sequence identities between BCoV isolates ranging from 97.9% to 99.8% and from 98.1% to 100%, respectively. Sequence alignment was used to create a phylogenetic tree (Fig. 1). Compared with BC94, we identified six amino acid substitutions in the HE genes of Korean field strains (0501, 0502, KCDs,

Figure 3. Phylogenic tree of spike proteins of Mebus, L9, F15, LY-138, ENT, vaccine strain M64668, Korean vaccine BC94, SUN5, 0501, 0502, KWDs, and KCDs were made using Clustal W multiple alignment method of MegAlign program in DNAstar.

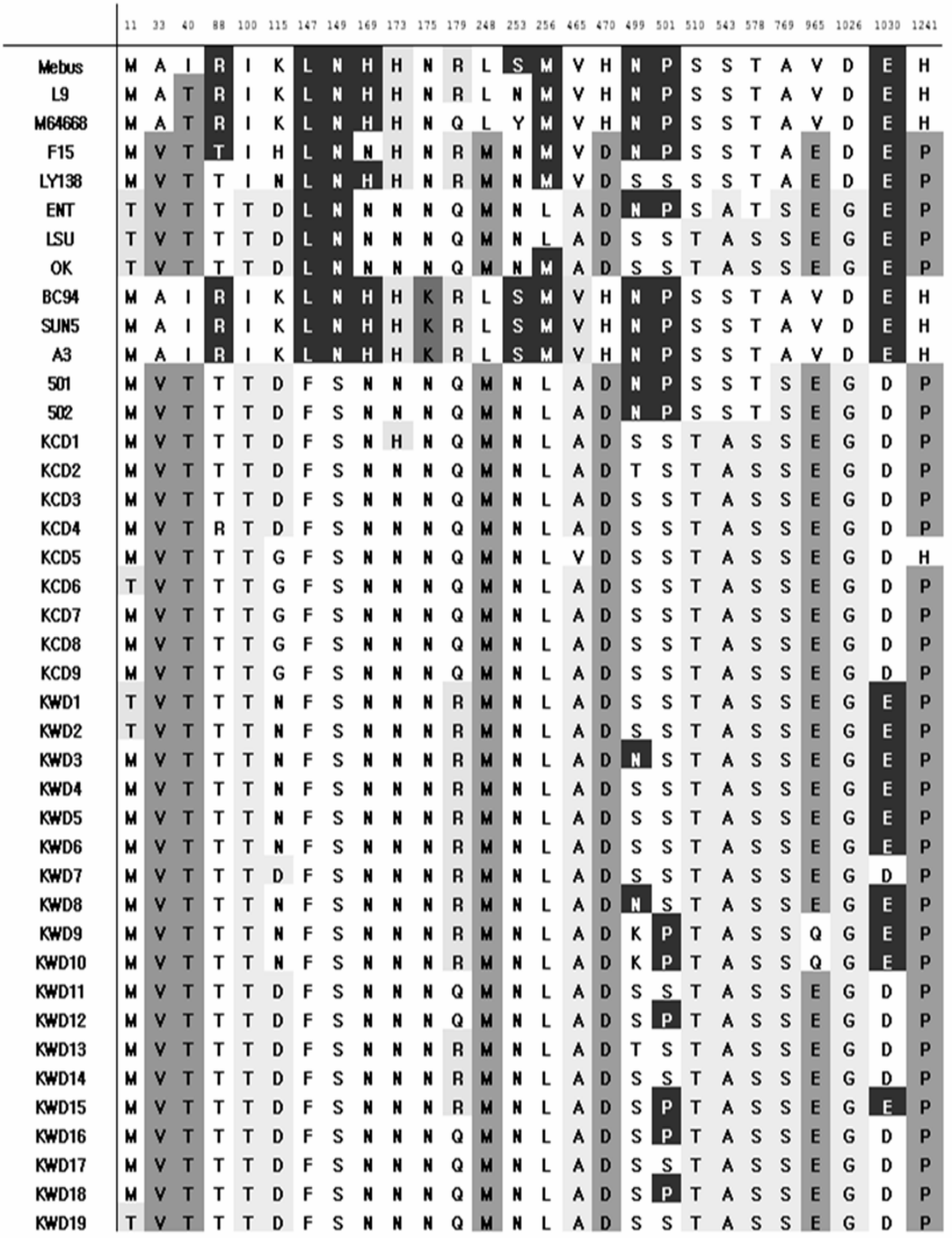

Figure 4. Comparison of the deduced amino acid sequences of hypervariable region in spike proteins. Light-gray boxes contain respiratory bovine coronavirus (RBCV)-specific; dark-gray boxes contain virulent-specific and black boxes show significant sequence compared to recent Korean wide-spread BCoVs.

KWDs) at L5P, C16S, D66G, H124Q, S367P, and L392I.

Molecular analysis of N gene

The N genes of the BC94 strain and four NVRQS strains each contained an ORF of 1347 nucleotides, encoding a predicted product of 449 aa residues. Pair-wise comparisons showed nucleotide and amino acid sequence identities between BCV isolates ranging from 98.0% to 99.9% and from 98.2% to 99.8%, respectively. Compared with BC94, we identified three amino acid changes (D77E, P162L, S423I) in the N gene. The phylogenic relationships between the N genes are shown in Figure 2.

Molecular analysis of S gene

The S genes of BC94 and four NVRQS strains each contained an ORF of 4092 nucleotides encoding a predicted product of 1346 aa residues. Sequence alignment analysis revealed nucleotide and amino acid sequence identities between BCV isolates ranging from 97.9% to 99.9% and from 97.9% to 100%, respectively. The phylogenetic relationships between S genes are shown in Figure 3. In total, 21 aa changes were detected in the S1 subunit (aa 1~768). Fifteen amino acid changes were detected in polymorphic regions in the N-terminal region of the S1 subunit (aa 1~ 330) (Fig. 4). The amino acid sequences of SUN5 and A3 strains were 100% identical to those of the BC94 strain. In the cases of 0501 and 0502, amino acids at positions 499, 501, 510, 543, and 578 were identical, but those at A33V, I40T, R88T, I100T, K115D, L147F, N149S, H169N, H173N, K175N, R179Q, L248M, S252N, M256L, V465A, and H478D were changed, compared with BC94. The amino acid sequences of 0501 and 0502 were similar to those of recent Korean isolates, KCDs and KWDs.

Cross reactivity of Korean vaccine strain and KCD9 strain

Postinoculation sera of guinea pigs inoculated with KCD9, the representative of recent Korean BCoVs, were tested to determine cross-reactivity to BC94 by a one-way virus neutralization (VN) test. The VN antibody titers against both KDC9 and the vaccine strain were determined in sera of all guinea pigs inoculated with KCD9 at postinoculation day 21 (PID21). The VN antibody titers were expressed as log2 values and did not differ significantly between the homologous and heterologous coronaviruses (Table 3), suggesting that KCD9 had cross-activity to BC94.

Table 3. Cross-reactivity of KCD9 and Korean vaccine strain determined by VN tests

BCV strain	VN antibody titers (log2)	
	KCD9	Korean strain
KCD9	6.00±1.15	6.67±0.52
Korean vaccine	5.75±0.96	6.17±0.98

DISCUSSION

BC94 has been used as a vaccine strain in calves in Korea since it was first isolated in 1994 without genetic characterization. In this study, HE, S, and N genes were sequenced to investigate the molecular features of BC94, and the genes were compared between vaccine and field strains.

Amino acid substitutions in the putative receptor-binding domain in the N-terminal region of the S gene can alter the tropism of the coronavirus (33). There were seven virulence-specific and thirteen respiratory BCoV (RBCV)-specific amino acid sites in the S gene (18).

In the present study, SUN5, A3, and BC94 had no virulence-specific sequence and one amino acid change, from asparagine to lysine at amino acid position 175 in the polymorphic region of the S gene, compared with Mebus. Strains 0501 and 0502 had virulence-specific sequences at all seven sites. The cleavage site of the spike genes, at 763~768 (KRRSRR), was conserved in all NVRQS isolates. Although the recently isolated virus and Korean vaccine strain were genetically different, the antigenic domain II of the spike protein at 528 amino acid position was not changed.

Amino acid changes in the hypervariable sites in strains and emergent years suggested a correlation. BCoV isolates during the period from 2002 to 2005 were changed at positions 88, 147, 149, 169, 256, and 1030. BC94 and four

NVRQS isolates had asparagine, proline, serine, serine, and threonine at aa positions 499, 501, 510, 543, and 578, respectively, as in Mebus and the reference vaccine strain (M64668), whereas KWDs, and KCDs had serine, serine, threonine, arginine, and serine, respectively, at these sites (Fig. 4).

Recent Korean isolates collected between 2002 and 2005 showed distinct genetic divergence from Korean isolates collected in 1994, including the vaccine strain.

Analysis of HE and S genes of KCDs and KWDs, which were isolated in 2002 and 2004, respectively, divided HE into four groups and the S protein into three groups (34). BC94, SUN5, and A3 strains were clustered with LY-138, LSU, Mebus, and L9, as group II, according to the HE genes. Strains 0501 and 0502 were clustered with OK, LUN, ENT, KCD8, and KCD10, as group III, according to the HE genes. BC94, SUN5, and A3 strains were clustered with F15, LSU, Mebus, and L9, as group III, according to the S genes. Strains 0501 and 0502 were clustered with OK, LY138, KCDs, and KWDs, as group I, according to the S genes.

To understand the immunoserological relationships between BC94 and field strains, a cross-reactivity assay was performed. KCD9 was chosen because it is a representative strain with the most common sequence among the field isolates, based on the results of sequence analysis.

The VN antibody titers of KCD9-inoculated antisera against homologous KCD9 and heterologous BC94 were not significantly different. These results indicated that BC94 is antigenically closely related to KCD9 and is still considered as an effective vaccine strain. Further studies are required to investigate cross-protection in calves.

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