

S1 glycoprotein gene analysis of infectious bronchitis viruses isolated in Korea

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Summary. Fifteen isolates of *Infectious bronchitis virus* (IBV) were obtained from the kidney, trachea, and cecal tonsil of IB suspected chickens between 2001 and 2002 years in Korea. The S1 glycoprotein gene of IBV isolates were amplified by reverse transcriptase – polymerase chain reaction (RT-PCR) and analyzed by restriction fragment length polymorphism (RFLP) analysis. Fifteen Korean IBV isolates were classified into 4 groups by their RFLP patterns using restriction enzymes, *Hae*III, *Bst*YI, and *Xcm*I. The RFLP patterns for 3, 1, and 1 of 15 isolates corresponded to the patterns of IBV Arkansas, Connecticut, and Massachusetts strains, respectively. Ten of 15 isolates generated unique KM91 RFLP pattern that was observed in the IBV KM 91 strain previously isolated in Korea. To confirm genetic diversity in the S1 genes of IBV isolates, viral RNAs of representative 9 of 15 IBV isolates were amplified, cloned, sequenced and compared with published sequences for non-Korean IBV strains. Korean IBV isolates showed amino acid sequence similarity between 61.8% (K446-01 and K161-02) and 96.1% (K281-01 and K210-02) with each other and they showed amino acid sequence similarity between 42.9% (K161-02 and GA980470) and 96.5% (K203-02 and KB8523) compared to non-Korean IBV strains. By phylogenetic tree analysis, Korean IBV field isolates were branched into five clusters in which 3 clusters were differentiated from non-Korean IBV strains. Especially, Korean IBV isolates K069-01, K507-01, K774-01 and K142-02 formed a separate cluster. It seems that IBVs continue to evolve and IBVs showing various genetic differences may cocirculate in Korea.

Introduction

Infectious bronchitis virus (IBV) is the etiological agent of infectious bronchitis (IB), which is an acute and highly contagious disease of the respiratory and

sometimes the urogenital tracts of chickens causing tracheal rales, sneezing, coughing, a poor weight gain and reduced feed efficiency in broilers and a decline in egg production and egg shell quality in layers [8].

IBV belongs to the family *Coronaviridae* [24]. It is a pleomorphic enveloped virus with club-shaped surface projections (spikes) on the surface of the virion and its genome consists of the single stranded positive-sense RNA genome of approximately 27 kilobases [1]. The virion contains four major structural proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein, the envelope (E) glycoprotein, and the nucleocapsid (N) protein [24, 30]. The S glycoprotein of IBV is posttranslationally cleaved into N-terminal S1 and C-terminal S2 subunits [7, 30]. The S1 glycoprotein forms the distal, bulbous part of the spike, and the S2 glycoprotein anchors the S1 glycoprotein to the viral membrane [3]. The S1 subunit is known to contain regions that induce neutralizing, serotype-specific, and hemagglutination-inhibiting antibodies [5, 18, 27].

A number of IBV serotypes and variants have been isolated and identified worldwide [10, 17]. These antigenic IBV variants do not completely cross-protect [13]. Therefore, IB continues to be an economically important disease to the poultry industry although IBV vaccines have been used to prevent IB outbreaks worldwide. Different serotypes and variants of IBV are thought to be generated by amino acid changes resulting from nucleotide insertions, deletions, or point mutations in the S1 subunit made by the viral polymerase [4, 20, 32].

Since IBV was first reported in 1986 in Korea and nephropathogenic IBV was recognized in 1990, various serotypes of IBV have been reported in Korea. And these IBV isolates showed different patterns from each other and non-Korean IBV isolates in reverse transcriptase-polymerase chain reaction-restriction fragment length polymorphism (RT-PCR-RFLP) analysis [29]. However, only Massachusetts (Mass) type live attenuated vaccines as well as inactivated oil-emulsion vaccines were used to control IB [16, 17, 28, 29].

The purpose of the present study was to genetically characterize IBV strains isolated recently in Korea. The S1 glycoprotein genes of the Korean IBV strains were amplified by RT-PCR. Amplified S1 genes were first classified by RFLP analysis and then the representative strains were cloned, sequenced and compared to other non-Korean published IBV sequences. By the RT-PCR-RFLP and phylogenetic analysis, recent Korean IBV isolates were classified into at least 5 different groups at the genetic level in which one group included only Korean IBV strains.

Materials and methods

Viruses

Field IBVs were isolated from kidney, trachea and cecal tonsil of IB suspected chickens using SPF embryonated eggs between 2001 and 2002 according to the standard procedure [11]. The history of these isolates is listed in Table 1. IBV isolates were propagated in 10-day-old specific pathogen free embryonated eggs and identified as IBV by IBV-specific RT-PCR as described below [22]. The harvested allantoic fluids were used to prepare viral RNA.

Viral RNA extraction and RT-PCR of S1 gene

The viral RNA was extracted from allantoic fluid as described previously [22]. Briefly, sodium dodecyl sulfate (final concentration, 2% wt/vol) and proteinase K (final concentration, 250 µg/µl) were added to the allantoic fluid and the mixture was incubated for 5 min at 55 °C. Viral RNA was extracted with acid phenol : chloroform (5:1, pH 4.7, Ambion, Woodward, U.S.A.) and chloroform : isoamylalcohol (IAA) (49:1), and further purified using the RNaid kit (BIO101, Carlsbad, U.S.A.). Finally, the RNA was resuspended in diethyl-pyrocabonate (DEPC) treated water and stored at -70 °C until used in the reverse transcriptase (RT) reaction.

Amplification of the S1 gene by RT-PCR was performed using the forward S1 OLIGO5' (5' TGAAAAGTGAACAAAAGA 3') and reverse S1 OLIGO3' (5' CTAAACTAACATAAGG GC 3') primer pair [22]. The RT reaction to synthesize cDNA contained purified RNA, 25 pmol S1 OLIGO3' primer and RT PreMix AccuPower RT PreMix (RTase, stabilizer and tracking dye) (BIONEER, Korea). The mixture was incubated at 42 °C for 60 min, and then heated for 5 min at 94 °C to stop the reaction. For the PCR reaction, 20 pmol each primer (S1OLIGO5' and S1OLIGO3') and cDNA were added to AccuPower PCR PreMix (Taq DNA polymerase, each dNTP, Tris-HCl, KCl, MgCl₂, stabilizer and tracking dye) (BIONEER, Korea). The PCR was performed by 35 cycles of denaturation at 94 °C for 90 sec, annealing at 45 °C for 30 sec, and polymerization at 72 °C for 90 sec. The final polymerization step was performed at 72 °C for 10 min. The PCR products were analyzed on a 1.0% agarose gel.

RFLP analysis

PCR products with the expected size (about 1720 bp) were excised from an agarose gel and purified using GeneClean kit (BIO 101). The restriction enzymes *Hae*III, *Bst*YI and *Xcm*I were used to digest the PCR products of S1 gene as described by Kwon et al [15, 23]. The RFLP patterns were observed after electrophoresis on a 2% agarose gel.

Cloning and sequencing

Nine representative IBV isolates (K069-01, K281-01, K446-01, K507-01, K774-01, K142-02, K161-02, K203-02, K210-02) of 15 isolates after RFLP analysis were sequenced. PCR products were cut from 1% agarose gels and purified using GeneClean (BIO 101). Purified PCR products were cloned into the TA cloning vector (Invitrogen, Carlsbad, CA) and transformed into competent cells (TOP10) (Invitrogen). Cells carrying recombinant plasmid were selected on LB agar plates containing ampicillin and X-gal. Plasmid DNA for sequencing was prepared by Plasmid Maxi Kit (Qiagen, Santa Clarita, CA, U.S.A.). Sequencing was performed with the M13 forward and M13 reverse primers. DNA sequencing was performed using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Sequence analysis

Nucleotide sequence data were compiled and analyzed using the Clustal V method in MegAlign software (DNASar, Inc. Madison, WI). Phylogenetic trees for S1 glycoprotein were generated using the maximum parsimony method with 100 bootstrap replicates in a heuristic search with the PAUP 4.0 software program (Sinauer Associates Inc., Sunderland, MA, U.S.A.).

The sequence data of S1 gene reported have been deposited in the GenBank database (Table 1). Sequences used for comparison or phylogenetic analysis in this study were obtained from the following GenBank database accession numbers: Arkansas 99 (M85244),

Table 1. History of Korean IBV field isolates, RFLP patterns, and GenBank accession numbers

IBV isolates	Organs ^a used for virus isolation	Production type ^b	Ages of IB outbreak (days)	Years of isolation	PCR-RFLP patterns	GenBank accession numbers
1. K069-01	K	B	18	2001	KM91 ^c	AY257061
2. K281-01	K	B	31	2001	Arkansas	AY257062
3. K434-01	CT	B	28	2001	Arkansas	. ^d
4. K446-01	T	B	21	2001	Connecticut	AY257063
5. K507-01	K	B	21	2001	KM91	AY257064
6. K748-01	K	B	37	2001	KM91	.
7. K774-01	CT	B	15	2001	KM91	AY257065
8. K044-02	K	B	60	2002	KM91	.
9. K058-02	T	B	70	2002	KM91	.
10. K117-02	CT	B	25	2002	KM91	.
11. K142-02	CT	B	28	2002	KM91	AY257060
12. K161-02	T	B	37	2002	KM91	AY257066
13. K203-02	T	B	35	2002	Mass41 + KM91	AY257067
14. K210-02	T	B	27	2002	Arkansas	AY257068
15. K234-02	T	B	16	2002	KM91	.

^aK = Kidney, T = Trachea, CT = Caecal tonsil

^bB = Broiler

^cKM91 was the representative isolate of Korean IBV isolates determined as genotype III which showed a distinct RFLP pattern in PCR-RFLP analysis [29]

^dDot (.) = That isolate was not sequenced

Arkansas DPI (AF006624), Beaudette (X02342), Connecticut (L18990), D41 (AF036937), DE072 (U77298), Florida (AF027512), GA980470 (AF274437), Gray (L14069), H120 (M21970), JMK (L14070), KB8523 (M21515), and Mass 41 (X04722).

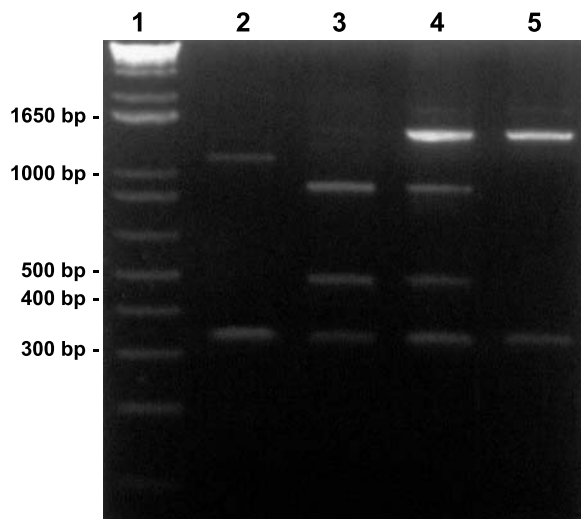


Fig. 1. RFLP patterns of the PCR-amplified S1 glycoprotein genes from Korean IBV isolates digested with restriction enzyme *Hae*III. 1 = 1 kb molecular weight maker (GIBCOBRL); 2 = K281-01; 3 = K446-01; 4 = K203-02; 5 = K507-01

Table 2. Comparison of the nucleotide and deduced amino acid sequences of the S1 glycoprotein gene of 9 Korean IBV isolates (K069-01, K281-01, K446-01, K507-01, K774-01, K142-02, K161-02, K203-02, and K210-02) and non-Korean IBV strains

	Percent similarity – Protein																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. K069-01	***	74.6	76.8	93.1	92.9	93.5	64.3	78.3	75.7	75.9	79.3	78.0	79.3	45.6	44.2	74.4	79.6	79.3	78.5
2. K281-01	76.8	***	72.6	74.5	74.0	73.3	64.2	73.7	96.1	79.6	76.5	74.9	74.7	44.7	43.6	75.3	75.2	75.2	73.7
3. K446-01	79.1	74.6	***	76.7	76.5	76.1	61.8	92.2	73.3	73.3	92.7	87.5	92.9	45.2	44.6	76.1	93.8	92.9	95.5
4. K507-01	96.1	76.5	78.8	***	97.2	91.6	63.6	78.2	75.8	75.5	79.1	78.4	79.3	45.8	45.3	74.5	79.7	79.3	78.1
5. K774-01	95.7	75.9	78.4	98.6	***	91.4	64.2	78.2	75.7	75.3	79.0	78.0	79.1	45.4	44.9	74.3	79.5	79.1	77.9
6. K142-02	96.6	76.2	78.0	95.6	95.4	***	63.8	77.1	74.3	74.3	78.2	77.4	78.2	45.2	44.2	72.8	78.6	78.2	77.0
7. K161-02	67.1	62.4	63.3	66.7	66.8	67.7	***	62.9	64.0	61.6	64.2	63.0	64.2	43.4	42.9	62.1	63.8	64.0	63.1
8. K203-02	79.8	74.9	95.6	79.6	79.3	78.9	63.1	***	74.9	74.5	94.8	88.7	96.5	46.4	45.9	77.3	98.0	96.5	92.6
9. K210-02	75.1	97.3	73.7	76.9	76.7	76.4	63.9	76.0	***	79.2	77.7	75.6	75.8	45.8	44.8	76.2	76.4	76.4	74.4
10. Ark DPI	73.6	78.9	76.0	75.6	75.5	75.0	63.6	75.7	78.0	***	79.6	74.9	75.6	45.0	44.0	79.7	76.3	76.4	75.1
11. Beaudette	79.9	76.0	96.6	80.0	79.5	79.5	64.1	96.6	77.1	77.0	***	88.9	95.0	46.7	46.3	79.1	96.1	95.2	93.1
12. Connecticut	75.4	73.9	93.6	74.4	73.9	78.5	62.9	92.9	68.2	74.5	93.8	***	89.1	46.6	45.4	76.8	90.4	89.7	89.1
13. D41	80.4	75.5	95.8	80.4	80.0	79.5	63.9	98.3	76.6	76.4	96.6	93.2	***	47.1	46.8	78.4	98.1	97.0	93.3
14. DE072	47.7	47.1	51.7	50.3	49.5	47.5	49.2	49.1	48.3	48.7	52.8	46.8	52.5	***	93.4	44.5	47.1	47.1	46.9
15. GA980470	49.3	47.5	49.2	50.2	51.0	49.3	49.6	49.4	48.6	48.6	51.4	47.0	49.5	96.0	***	43.4	46.6	46.4	46.1
16. Gray	74.5	75.4	77.7	75.6	75.2	74.9	59.6	79.1	75.9	81.3	79.8	77.8	80.8	47.2	46.9	***	78.8	78.4	78.3
17. H120	80.5	75.6	96.3	80.2	79.8	79.5	63.8	98.9	76.7	76.4	97.3	93.7	99.1	52.0	49.5	80.9	***	98.1	94.2
18. KB8523	80.3	75.8	95.9	80.1	79.8	79.4	64.1	98.1	76.8	76.7	96.8	93.3	98.5	52.4	49.6	80.8	98.9	***	93.3
19. Mass41	78.9	75.0	96.6	79.6	79.2	77.8	64.0	95.6	75.3	77.1	96.6	93.7	95.8	49.5	46.2	80.5	96.3	95.9	***

Percent similarity – Nucleotide

Results

Virus isolation and RT-PCR-RFLP

Fifteen IBVs were isolated from trachea, kidney and cecal tonsil of broiler chickens (Table 1). Six, 5 and 4 of 15 IBVs were isolated from trachea, kidney, and cecal tonsil respectively. The age of chickens with IB outbreaks was from 15 to 70 days.

RT-PCR-RFLP analysis was initially performed to classify IBV isolates. Fifteen Korean IBV isolates were classified into four groups by RFLP analysis (Fig. 1). The RFLP pattern for isolate K446-01 corresponded to a pattern for IBV Connecticut strain. Isolate K203-02 showed both Massachusetts and KM91 RFLP patterns. Three isolates (K281-01, K434-01, and K210-02) were identical to the IBV Arkansas strain and 10 isolates (K069-01, K507-01, K748-01, K774-01, K044-02, K058-02, K117-02, K142-02, K161-02 and K234-02) had same RFLP pattern as the IBV KM91 strain which was isolated in 1991 in Korea [29].

Sequencing and sequence analysis

The whole S 1 gene of representative 9 of Korean IBV isolates was sequenced to further characterize the isolates. The nucleotide and deduced amino acid sequences of those IBV isolates were determined and compared with the sequences of published non-Korean IBV strains (Table 2, Fig. 2).

Korean IBV isolates had nucleotide sequence similarity between 62.4% (K281-01 and K161-02) and 96.6% (K069-01 and K142-02) with each other and they had nucleotide sequence similarity between 47.1% (K281-01 and DE072) and 98.9% (K203-02 and H120) with non-Korean IBV strains. Korean IBV isolates had amino acid sequence similarity between 61.8% (K446-01 and K161-02) and 96.1% (K281-01 and K210-02) with each other and they had amino acid sequence similarity between 42.9% (K161-02 and GA980470) and 96.5% (K203-02 and KB8523) compared to non-Korean IBV strains.

The Korean IBV K446-01 isolate had an S1 amino acid sequence most similar to the Mass41 strain (95.5%) and 93.8% similar to the H120 strain. Korean IBV isolate K203-02 had an S1 amino acid sequence most similar to H120 (98.0%) and 92.6% similar to Mass41. Isolates K281-01 and K210-02 had amino acid sequences similar (79.2%–79.6%) to the Ark DPI strain. Isolates K069-01, K507-01, K774-01 and K142-02 were 91.4% to 97.2% similar to one another, and they were 72.8% to 79.7% similar to eight published non-Korean strains (Ark DPI, Beaudette, Connecticut, D41, Gray, H120, KB8523, and Mass41). Isolates K161-02 had an S1 amino acid sequence 61.8% to 64.3% similar to other Korean isolates and 61.6% to 64.2% similar to 8 published non-Korean strain (Ark DPI, Beaudette, Connecticut, D41, Gray, H120, KB8523, and Mass41).

The deduced amino acid sequences of Korean IBV isolates were aligned with the sequences of published non-Korean IBV strains (Fig. 2). The most variations were observed between residues 53–96, 115–152, and 376–392 (numbering is in reference to Mass41 strain).

K069-01	MLGKSLFLVT	ILCALCSAN*	****NNYVY	YQSFRPPTG	WHLQGGAYAV	VNSTNYTNA	GSATACTVGV	IKDVYNQSA	75
K281-01	--VR--V--	--F-----L	YD*NGTF--	-----SG-	--H-----	--VSTEF--	--NRE--A-A	-YWSK-F--S	79
K446-01	--VTP-L---	L-V-----AL	YD*SSS---	-----N-	--H-----	--ISSES---	--SPG-I--T	IHGGRVVN-S	79
K507-01	-S--L-----	--Y-----L	VDFH**---	-----NV	-----H---	--GTSE---	-----	-----	78
K774-01	-S--L-----	-----L	VDFH**---	-----N-	-----	--GASE---	-----	-----	78
K142-02	-----M---	-----L	VDVNNN---	-----	-----K--	--HSS---	--SE---	-----	80
K161-02	--RVHL--VL-	-----L	FDSGDT-A--	---Q---S-	--QH---K-	--STFSN-SQG	****K-VL-S	-NYGHTLNV	76
K203-02	--VTP-L---	L-----VL	YD*SSS---	D-----D-	--H-----	--ISSES---	--SSG---I	-HGGRVVN-S	79
K210-02	--VRP--V--	--F-----L	YD*NA*F---	-----SG-	--H-----	--VSTEF--	--NSE--A-A	-YWSK-F--S	78
Ark DPI	--V-----	--F-----L	YD*NESF---	-----GH-	--H-----	--VSEEN---	--T-PS--A-A	-GYSK-L--A	79
Beau	--VTP-L---	L-----VL	YD*SSS---	-----S-	-----	--ISSEF---	--SSG---I	-HGGRVVN-S	79
Conn	--VTP-L---	L-F-----AL	YD*NSS---	-----N-	--H-----	--TFIES--*	**LRE-I--I	-GGDRVVN-S	75
D41	--VTP-L---	L-----AL	YD*SDS---	-----D-	--H-----	--ISSES---	--SSG---I	-HGGRVVN-S	79
DE072	--A-----I--	L-L---EGGL	VG**E--T--	---EY---N-	--M-----K-	--K-TISY**	*TNQ--I--	-GGVTTINQS	75
GA980470	--V---I---	L-L---EGGL	VG**V--T--	---GY---N-	--M-----	--K-TISY**	*TNQE--I--	-RGGVTTINQS	75
Gray	--VTP-L---	L-L---G--SL	LN*NDS---	-----N-	--H---K-	--VSEY---	PGNSG-VA-A	-FWSK-F--	79
H120	--VTP-L---	L-----AL	YD*SSS---	-----D-	--H-----	--ISSES---	--SSG---I	-HGGRVVN-S	79
KB8523	--VTP-L---	L-L---AL	YD*SSS---	-----D-	--H-----	--ISSES--E	--SSG---T	-HGGRVVN-S	79
Mass41	--VTP-L---	L--V---AL	YD*SSS---	-----N-	--H-----	--ISSES---	--SPG-I--T	-HG-VYVN-S	79
K069-01	SIAMTAPLQW	MAWPKSQFCS	AHCNFSEITV	FVTHCYSRGS	GSCPITGMIP	RDHIRIS***	****AMKNGS	LFYNLTVSVS	148
K281-01	-----SI-	-S-STRE--T	-----N-V-	-----FKS--	DY--L--L-Q	SGLV-V**SA	MKIGGSGPSD	-S-----P-T	157
K446-01	-----SS-	---SS---T	-----DT--	-----KY**	DG-----LQ	KNFL-VS***	*****Q	-----A	150
K507-01	-----S---	-----	-----	-----S-R	-----L-	QN-----	*****	-----	151
K774-01	-----S---	-----	-----	-----S-M	-----R-	QNY-----	*****	-----	151
K142-02	-----H---	--T--V--	--S-----	-----S--	-----	QN-----	*****	-----P	153
K161-02	AV-I---S--	-S-SATSL-T	VY---DF--	-I---*TGAG	NA-KL--DL*	*****GVGN	LRVSS-R--V	-IF-Y-LITV	148
K203-02	-----SS-	---SS---T	-Y-----	-----KH**	VG-----LQ	QHS--V-***	*****Q	-----A	150
K210-02	-----SI-	-S-STR---T	-----N-V-	-----FKT--	-Y--L--L-Q	SGLV-V**SA	MKIGGSGPSD	-----P-T	156
Ark DPI	-V-----S-	-S-SANS--T	-----TSYI-	-----KS-	N--L--L-	SGY---**AA	MKHGSAMP-H	-----T	157
Beau	-----SS-	---SS---T	-----DT--	-----KH**	-G--L--LQ	QNL--V-***	*****Q	-----A	150
Conn	-----QP-	-G-SSR--T	-----D--	-----KH**	NG-----S-	QHS--V-***	*****K-R	-----N	146
D41	-----R--	---SN---T	-Y-----	-----KQ**	-G-----LQ	QHS--V-***	*****Q	-----S-A	150
DE072	A--FASA*I-	IE-SKQGV-T	-Y--YTSFY-	-----**G-T	-HSC-VNTQQ	TGVLVFGVKN	Y*****DQ	FI--R-LVAA	146
GA980470	A--F-SA*T-	SS-S-QGV-T	VY--YTSFY-	-----**G-T	-HNC-VNTQQ	TGVLVFGVKN	Y*****DQ	FI--R-LGAA	146
Gray	-V--I--HS-	-S-SVQ--T	-----THF-	-----FKV-A	-L--L--PLL	QGG--**SA	MRSVNSRP*H	-----T	156
H120	-----SS-	---SS---T	-Y-----	-----KH**	VG-----LQ	QHS--VS***	*****Q	-----A	150
KB8523	-----SS-	-D-SS---T	-Y-----	-----KH**	-G-----LQ	QHS--V-***	*****Q	-----D	150
Mass41	-----SS-	---SS---T	-----DT--	-----KH**	-G-----TLK	QHT--V-***	*****Q	-----A	150
K069-01	*TYPNFKSLQ	CVNNFTSVYL	NGDLVFTSNK	TTDVTASAGVY	FKAGGPNVYN	IMKEFKVLAY	FVNGTVPDVI	LCDDTPRGLL	227
K281-01	*K--K-R-F-	-----H----	-----E	-Q--SA--H	-----IT-K	V-R-V-A---	-----A----	---G-K----	236
K446-01	*K--T--F-	-----L----	-----Y--E	-----	-----IT-K	V-R-L-A---	-----A----	---GS----	229
K507-01	*K-----F-	-----	-----	-----	-----T--	-----	-----	-----	230
K774-01	*K-----F-	-----	-----	-----	-----T--	-----	-----	-----	230
K142-02	*K-----F-	-----L----	-----	-----	-----R-	-----	-----	-----	232
K161-02	*AH-K---LH	-----	-----I	-A--SA--A-	--SS--K-K	T-QQYN---	-E--GH--	--KS-K---	227
K203-02	*N--T--F-	-----L----	-----Y--E	-----	-----T--	V-R-VRA---	-----A----	---GS----	229
K210-02	*K--K-R-F-	-----H----	-----C--	-Q--SA--H	-----T--	V-R-V-A---	-----A----	---G-K----	235
Ark DPI	*K--K-R--	-----Y----	-----Y	-E--VA--H	-S---IT-K	V-R-V-A---	-----AH----	-----	236
Beau	*K--T-R-F-	-----L----	-----Y--E	-----	-----IT-K	V-R-V-A---	-----A----	---GS----	229
Conn	*K--T--F-	-----L----	-----Y--E	-----	-----N---	IT-K V-R-V-A---	-----A----	---GS----	225
D41	*K--T--F-	-----L----	-----Y--E	-----	-----IT-K	V-R-VRA---	-----A----	---GS----	229
DE072	GV-A--AW-	-LS-Y--F-	-N-LY--Y	-E--KA----	A-QVNGLERR	V-RDTPVMAY	-----	---SPK-RL	236
GA980470	GP-A--AW-	-LS-Y--F-	-N-LY--Y	-E--KA----	A-QVNGLERR	V-RDTP-M--	-----	---S-K-R-	226
Gray	*K--L-----	-----Q----	-----S-E	-I--SG--H	-----IT-K	V--T-A---	-----A----	---GS----	235
H120	*K--T--F-	-----L----	-----T--E	-----	-----IT-K	V-R-VRA---	-----A----	---GS----	229
KB8523	*K--T--F-	-----L----	-----Y--E	-----	-----V--	IT-K V-R-VRA---	-----A----	---GS----	229
Mass41	*K--T--F-	-----L----	-----Y--E	-----	-----IT-K	V-R-V-A---	-----A----	---GS----	229
K069-01	ACQYNTGNFS	DGFYPTNSS	LVKQRFVYR	ESSVNTTLTL	TNFTFHNETN	ASPNS*GSVH	SISTYQTQTA	QSGYYNFNLS	306
K281-01	-----	-----	-----EK-I---	-N-----V-	H-V-S-VSG-	T---*G-D-	-QI--H---	-----Y-F-	315
K446-01	---ILAI-Q	MAL*S-I---	-----K-I---	-N-----F--	H-----G-	N--P*S-VQ	N-Q-----	-----F-	308
K507-01	-----	-----	-----I--H	-----	-----	---*G-N	-----A-	-----	309
K774-01	-----	-----	-----I---	-----	-----V-----	---*G-N	-----R-	-----	309
K142-02	---LS---	-----	---E---	-----	-----	---L*-G-N	T-----	-----	311
K161-02	-----	-----LIPH	SLRKNLLFFW	-NN-TEY-V-	H-VS---QS-	-V--T*-G-D	T-LK--H--	-D-----Y-F-	306
K203-02	-----	-----K-I---	-N-----F--	H-----G-	N--P*SG-Q	N-Q-----	-K--KILIFP	-----	308
K210-02	-----	-----EK-I---	-N-----V-	H-V--S-VSG-	T--S*-G-G	-QI--H--	-----Y-F-	-----	314
Ark DPI	-----	-----T-	I--DK-I---	-----S--SG	-P--T*-G-D	SFIL-----	-----F-	-----	315
Beau	-----	-----K-I---	-N-----C-	H--I-----G	N--P*SG-Q	N-Q-----K-	-----F-	-----	308
Conn	-----	-----K-I---	-N--I---K-	H-----G-	N--L*SG-Q	N-Q-----	-----F-	-----	304
D41	-----	-----K-I---	-N-----F--	H-----G-	N--P*NG-Q	N-Q-----	-----F-	-----	308
DE072	--L--VYEEP	VASNFTF-PL	DI--STSYGV-	H---N-V-G	VE--GK***	H-ARFNIS-I	PE--V--KFN	-----	302
GA980470	--L--VYEEP	VASNFTF-SL	DT--STSYGV-	H---N-V-G	VA--QN***	Y-ARFNIS-I	SE--V--KFN	-----	302
Gray	-----GT	I--DR-I---	-T-----	T-----R--SA	-P--N*-G-D	TFIL-----	-N-----F-	-----	314
H120	-----	-----K-I---	-N-----F--	H-----G-	N--P*S--Q	N-Q-----	-----F-	-----	308
KB8523	-----	-----K-I---	-N-----F--	H-----G-	N--P*SG-D	N-Q-----	-----F-	-----	308
Mass41	-----	-----I---	-----K-I---	-N-----F--	H-----G-	N--P*SG-D	N-Q-----	-----F-	308

Fig. 2 (continued)

K069-01	FLSSFYKES	NFMYGSYHP*	**S*CNFRLE	TINNGLWFNS	LSISLAYGPL	QGGCKQSVFS	SRATCCYAYS	Y*NGPRACKG	381
K281-01	---S-RQ-	D-----*	**K*-S-KP-	-L--L----	-T--LMAHS	-----N	N-----T	-*S--H----	390
K446-01	-----*	-----*	**--*	-----*	---V-I----	-----*	G-----*	*G--SL---	383
K507-01	-----*	-----*	**--*	---K-	-----*	-----*	-K-----*	-*N-----	384
K774-01	-----*	-----*	**--*	-----*	-----*	-----*	-K-----*	-*-----*	384
K142-02	-----*	-----*	**--*	---P-	--K-----*	-----*	-----*	-*-----*	386
K161-02	-----V-	D-T-----*	**--LVI--P-	---I-----*	-----G----	R-----T	GK-S-----*	-*---SI---	381
K203-02	-----C--*	-----*	**--*	---L-	-----*	---V-I----	G-----*	*G--LL---	383
K210-02	---S-RK-	D-----*	**K*-S--P-	-L-----*	---TI-L----	-----N	N-----*	-*S--HA---	389
Ark DPI	-----R--	-Y-----*	**R*-S--P-	-L-*-----*	---V-LI--I	-----N	GK-----*	-*G--A---	390
Beau	-----*	-----*	**--*K-	-----*	---V-I----	-----S	G-----*	*G--SL---	383
Conn	---G-F---	-----*	**--*	---P-	-----*	---V-I----	G-----*	*G--SL---	379
D41	-----*	-----*	**--*	---L-	-----*	---V-I----	G-----*	S*G--LL---	383
DE072	--N--T-V--	D-DR---YGK	PG-R---P-	S--R--S---	-TV-IG--I	S-----WQ	NE---F-K-	-N-GSRN---	382
GA980470	--N--T-V--	D-DR---YGK	PG-R---P-	S--R--S---	-TV-IG--I	-----*	G-----*	*G--SL---	381
Gray	---D---A-	D-----*	**H*-S--P-	-L-----*	---V-IT----	-----*	G-----*	*R--SL---	389
H120	-----*	-----*	**--*	-----*	---V-I----	-----*	G-----*	*G--LL---	383
KB8523	-----*	-----*	**--*S-	-----*	---V-I----	-----*	G-----*	*G--LL---	383
Mass41	-----*	-----*	**--*	---S-	-----*	---V-I----	G-----*	-*E--SL---	383
K069-01	VYAGELHQS	ECGLLVYTK	SDGSRIQTAT	EPPVITQHN	NNITLNTCVD	YNIYGRIGQG	FITNVTSS	SYNYLADAGS	461
K281-01	--T--T-N-	-----I--	-----*	-A--L--NF-	-----K--E	-----V--	-----T	TF---E-G--	470
K446-01	--S---DLN-	-----*	-G-----*	-----R--	-----*	-----T--	-----AV	-----L	463
K507-01	-----S--	-----*	-----*	-----*	-----*	-----R-	-----*	R-----L	464
K774-01	-----S--	-----*	-----P	--LG----	-----*	-----R-	-----*	R-----L	464
K142-02	-----Q--	-----*	-----*	-----I--	-----*	-----*	-----*	-----L	466
K161-02	-----K--	---FI-S-	-A-----SN	TV-TF-RF--	-----K--	-----V--	H-V---AAK	D-----L	461
K203-02	-----DHN-	-----*	-G-----*	-----*	-----*	-----*	-----AV	-----L	463
K210-02	--T--T-N-	-----I--	-----*	-A--L--NF-	-----K--E	-----V--	-----TS	TF---E-G-L	469
Ark DPI	--R---T-H-	-----*	-----*	Q--L--NF-	---N-GK--	-----*	L-----LAV	---S--L	470
Beau	--S---DHN-	-----*	-G-----*	-----N-	-----*	-----*	-----AV	-----L	463
Conn	--L---KSD-	-----*	-----*	-----*	-----*	-----*	-----AV	-----M	459
D41	--S---DHN-	-----*	-G-----*	-----H--	-----*	-----*	-----AV	-----L	463
DE072	--NFDKDVNY	--V---FIS-	T-----R--	S---Y--NN-V	I--S--GL--	-----*	-----AV	-----L	462
GA980470	L-TFSDVNY	--V---FIS-	T-----R--	S---YSNN-V	*--N--GL--	--V--IT-R-	L---I-E-VH	P*G--DHG-L	459
Gray	--S---LRN-	-----*	-Q-----*	-----Y--	-----*	-----*	-----LAV	-----L	469
H120	--S---DHN-	-----*	-G-----*	-----*	-----*	-----*	-----AV	-----L	463
KB8523	--S---DHN-	-----*	-G-----*	-----*	-----*	-----*	-----AV	-----L	463
Mass41	--S---DLN-	-----*	-----*	-----R--	-----*	-----*	-----LAV	-H-----L	463
K069-01	AILDTSGAID	IFVVQGEHGL	NYYKVNPCED	VNQQFNNSGG	KLVGILTSRN	ETGSQPLENQ	FYIKLTKETR	RFRR	535
K281-01	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	544
K446-01	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537
K507-01	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	538
K774-01	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	538
K142-02	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	540
K161-02	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	535
K203-02	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537
K210-02	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	543
Ark DPI	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	544
Beau	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537
Conn	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	513
D41	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537
DE072	VL--AT-S--	T--LHRDHLT	S-----S-	I-E-Y----	N---K--N-	Q-VA-Q-GDM	--V-FSTSS-	-I--	536
GA980470	VL--AT-S--	T--LHSDNLT	S-----S-	I-E-Y----	N---K--N-	Q-VA-Q-GDM	--V-FSTSG-	-I--	533
Gray	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	543
H120	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537
KB8523	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537
Mass41	-----S--	-----Y--	T-----*	-----*	-----*	-----L--	-----I--NG--	-----S--	537

Fig. 2. The deduced amino acid sequence comparisons of the S1 glycoprotein gene of 9 Korean IBV isolates (K069-01, K281-01, K446-01, K507-01, K774-01, K142-02, K161-02, K203-02, and K210-02) and 10 published non-Korean IBV strains. The dashes(-) indicate regions where the sequences are identical to those of K069-01. Deletions within the sequences are shown with asterisks (*). Sharps (#) indicate unavailable sequence

Phylogenetic trees were constructed from the nucleotide and deduced amino acid sequences of the S1 glycoprotein genes of Korean IBV isolates and non-Korean IBV strains (Fig. 3). Nine Korean IBV isolates were grouped into five distinct branches. The K446-01 isolate formed the first branch in which Mass41 was included. The K203-02 isolate formed the second branch that related to non-

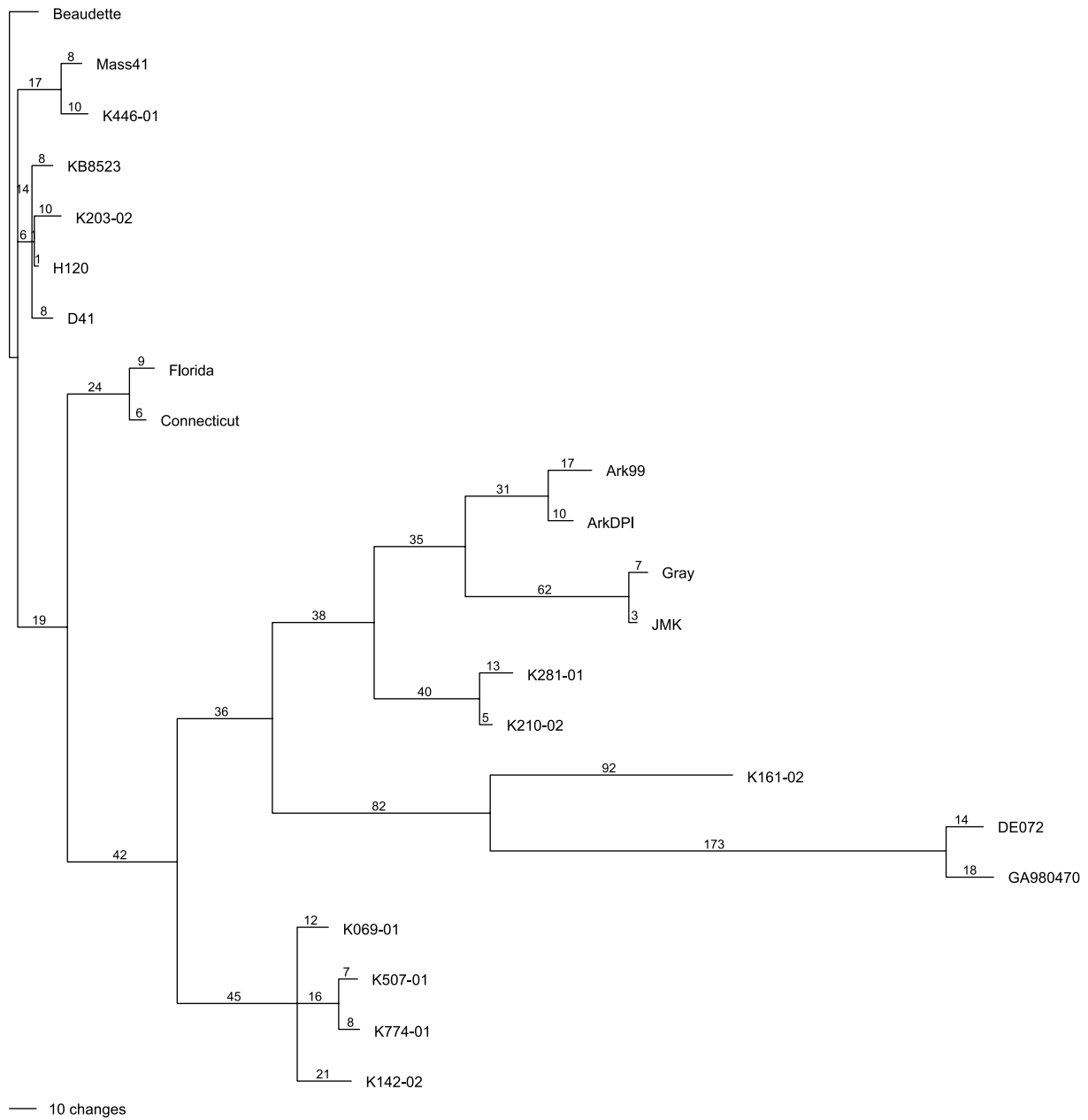


Fig. 3. Phylogenetic relationship based on the deduced amino acid sequences of the S1 glycoprotein of the Korean IBV field isolates (K069-01, K281-01, K446-01, K507-01, K774-01, K142-02, K161-02, K203-02, and K210-02) and non-Korean IBV strains generated by the maximum parsimony method with heuristic search and 100 bootstrap replicates. The tree was rooted to a sequence of the IBV Beaudette strain. The length of each branch represents the number of amino acid changes between sequences

Korean IBV isolates, KB8523, H120, and D41. The K281-01 and K210-02 isolates formed the third branch and the K161-02 isolate formed the fourth branch. K069-01, K507-01, K774-01 and K142-02 isolates formed the fifth independent group.

The spike glycoprotein of IBV is translated as a precursor protein (S_0), and then cleaved into two subunits S1 and S2 [7, 22]. The IBV spike glycoprotein cleavage recognition site for Korean IBV isolates K069-01, K446-01, K507-01, K774-01, K142-02, K161-02 and K203-02 isolates had the sequence Arg-Arg-Phe-Arg-Arg, which was found in the published IBV Beaudette, D41, H120, KB8523, and Mass41 strains [7, 14]. Whereas Korean IBV isolates K281-01 and K210-02 isolates had the sequence Arg-Arg-Ser-Arg-Arg which was found in the published IBV Ark DPI and Gray strains [14, 22].

Discussion

IB has been a continual problem in Korea although both Mass type live attenuated vaccine and inactivated vaccine were widely used to control the disease. Fifteen Korean IBV isolates were initially analyzed by RT-PCR-RFLP and followed by nucleotide sequencing of the S1 glycoprotein gene in this study.

Korean IBV field isolates between 1986 and 1997 were characterized using RT-PCR-RFLP analysis and pathogenicity testing but the sequences of those viruses were not reported [29]. According to RT-PCR-RFLP analysis, 40 IBV field isolates were classified into five genotypes (I, II, III, IV, and V). Six genotype I IBV isolates showed similar RFLP patterns to Mass type of IBV and only 2 genotype II IBV isolates were only isolated in 1986. Twenty-nine genotype III IBV (KM91 type) isolates showed distinct RFLP patterns in PCR-RFLP analysis using restriction enzymes *Hae*III, *Eco*RI, and *Bam*HI [29]. The KM91 isolate is the representative genotype III isolate. Genotypes IV and V were newly isolated in 1995. In this study, 10 of 15 field IBV isolates were classified as the KM91 type and 3 isolates were classified as Arkansas and 1 as Connecticut types. In pathogenicity tests, isolate KM91 caused 50% mortality, severe nephritis and renal urate deposition in the kidneys of infected chicks, but genotypes I, II, IV and V only induced respiratory distress at 1 to 2 days after inoculation [29]. The H120 vaccine could not protect chicks against challenge with the KM91 isolate, genotype III. Therefore, the KM91 type has seemed to be a major IBV circulating in Korea. The Korean IBV K203-02 isolate showed both Massachusetts and KM91 type RFLP patterns in PCR-RFLP analysis. The detection and differentiation of two different kinds of viruses in a single sample is a significant advantage of RT-PCR-RFLP analysis which other researchers have also reported [2].

Korean IBV isolates (K069-01, K507-01, K774-01, K142-02 and K161-02) sequenced among IBV isolates classified as KM91 type by RFLP analysis showed 63.4% to 97.2% nucleotide sequence similarity and 66.7% to 98.6% amino acid sequence similarity among themselves. It seemed that IBVs with differences in genetic composition existed in field condition although they showed identical RFLP patterns.

By alignment of the S1 glycoprotein of Korean IBV isolates with published non-Korean IBV strains, the most sequence variations were observed between residues 53–96, 115–152 and 376–392. Regions between 53–96 and 115–152 showing high amino acid variations herein were similar to hypervariable region 1 (HVR 1, residues 56–69) and hypervariable region 2 (HVR 2, residues 117–131) of IBV [6, 20]. The HVRs are associated with two separate viral neutralizing and conformationally dependent epitopes [6, 20]. Amino acid variation region 376–392 was similar to region III (274–387) associated with a neutralizing epitope [19]. Virus neutralization tests will be needed to definitively classify Korean IBV isolates into a distinct serotype although PCR-RFLP pattern correlates with serotype [23].

In the phylogenetic tree, Korean IBV isolates formed five distinct clusters. Korean IBV K446-01 isolate was clustered into Mass41 group although it was classified into the Connecticut group by PCR-RFLP analysis. Korean IBV K203-02 isolate was clustered into the same genotypic clade as non-Korean IBV strains KB8523, H120, and D41 that were an attenuated vaccine strains [21, 26, 31]. Therefore, K203-02 isolate appears to be originated from a vaccine strain. Korean IBV K281-01 and K210-02 isolates formed distinct clusters that were related to non-Korean IBV Ark99, Ark DPI, Gray and JMK strains although K281-01 and K210-02 isolates were classified into the Arkansas type by PCR-RFLP analysis. Two Korean IBV isolates K281-01 and K210-02 had the same Arg-Arg-Ser-Arg-Arg on spike glycoprotein cleavage recognition sites as the non-Korean IBV strains Ark DPI, Gray, and JMK [14]. Spike glycoprotein cleavage recognition site does not appear to correlate with serotype or pathogenicity [14]. Korean IBV K161-02 isolate formed a distinct cluster although it was classified into the KM 91 type with other several IBV isolates by PCR-RFLP analysis. It showed 61.8% to 64.3% amino acid sequence similarity compared to other Korean IBV isolates, which was lower similarity than other IBV isolates. It was noteworthy that K161-02 isolate was related to non-Korean IBV isolate DE072 and GA980470 in the phylogenetic tree although it showed 42.9% (K161-02 and GA980470) to 43.4% (K161-02 and DE072) amino acid sequence similarity. The IBV DE072 strain was involved in IB outbreaks of broilers on the Delmarva peninsula, U.S.A. in 1992, and it was classified into the Delaware variant (DE var) type by virus-neutralization tests, cross-challenge tests and S-1 gene analysis [12]. The IBV GA980479 strain was isolated from broilers in Georgia, U.S.A. and is similar to the IBV DE072 strain [25]. But it was genetically distinct from IBV DE072 and shared very low antigenic relatedness with IBV DE072 and other IBV isolates and thus was designated into a new serotype, Georgia 98 [25]. It seems that the Korean IBV K161-02 isolate may be one of the Korean IBV variants. Further characterization by virus-neutralization tests and cross-challenge tests using several IBV strains will be needed. The Korean IBV K069-01, K507-01, K774-01, and K142-02 formed a distinct cluster that consists of only Korean IBV isolates. They were classified into the IBV KM91 type by PCR-RFLP analysis, which has been isolated mainly in Korea [29].

The evolution of IBV is seemed to be influenced by a number of factors such as the use of multiple strains for vaccination, population density and host

immune status [9]. In addition, IBV has a high error rate during the genomic transcription and then, produces a quasispecies phenomenon where many different viral genotypes will cocirculate in the host, with each virus potentially having different levels of fitness for the host environment [9, 24]. Widespread uses of various vaccines made from heterologous IBVs in the field may play an important role in increasing the number of new genetic variants in Korea.

In conclusion, 15 Korean IBV field isolates were different from published non-Korean IBV strains in nucleotide and amino acid sequences and were clustered into different groups from non-Korean IBV isolates by phylogenetic tree analysis. And 15 Korean IBV isolates were clustered into at least 5 groups in which 3 groups were differentiated from non-Korean IBV isolates. Virus-neutralization test and cross-challenge tests using several IBV strains will be needed to further characterize the IBV isolates.

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