Genomic characteristics of natural recombinant infectious bronchitis viruses isolated in Korea

Hyun-Woo Moon, Haan Woo Sung, Hyuk Moo Kwon*

*Laboratory of Veterinary Microbiology, College of Veterinary Medicine and Institute of Veterinary Science,
Kangwon National University, Chuncheon 24341, Korea*

Abstract: Two infectious bronchitis virus (IBV) K046-12 and K047-12 strains were isolated and the nearly complete genomes of them were sequenced. Sequence comparisons showed that the K046-12 genome was most similar to Korean IBV strains, and the K047-12 genome was most similar to QX-like IBV strains. Phylogenetic analysis showed that nearly all K046-12 and most K046-12 genes were placed in the same cluster as Korean IBV isolates, but the S1 region was placed in the same cluster as Mass-type IBVs. For K047-12, nearly all K047-12 and most K047-12 genes were located in the same cluster as QX-like IBVs, but the M region was located in the same cluster as Korean IBV isolates with K047-12. Recombination analysis confirmed that K046-12 is a recombinant strain with the primary parental sequence derived from Korean IBVs and minor parental sequence derived from Mass-type IBV, and K047-12 is a recombinant strain with the major parental sequence derived from QX-IBV and minor parental sequence derived from Korean IBVs. This study showed that new IBV recombinants are constantly generated among various IBVs, including those used for vaccination. Therefore, genetic analysis of new virus isolates should be performed for effective infectious bronchitis control and appropriate vaccine development.

Keywords: infectious bronchitis virus; recombinant; sequence; phylogenetic trees

*Corresponding author

Hyuk Moo Kwon
Laboratory of Veterinary Microbiology,
College of Veterinary Medicine and
Institute of Veterinary Science, Kangwon
National University, Chuncheon 24341,
Korea

Tel: +82-33-250-8652
Fax: +82-33-254-5625
E-mail: kwonhm@kangwon.ac.kr

ORCID:
Hyun-Woo Moon
<https://orcid.org/0000-0002-4191-7590>
Haan Woo Sung
<https://orcid.org/0000-0001-7715-1390>
Hyuk Moo Kwon
<https://orcid.org/0000-0003-4953-8293>

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Introduction

Infectious bronchitis (IB) is an acute and highly contagious disease in chickens, causing respiratory stress and urogenital tract diseases [1]. IB virus (IBV), the etiologic agent of IB, is an enveloped, positive-sense, single-stranded, non-segmented RNA virus with a genome of approximately 27.6 kb [2,3]. IBV possesses at least 10 open-reading frames (ORFs): 5' untranslated region (UTR)-1a-1b-S-3a-3b-E-M-5a-5b-N-3'UTR [4]. The replicase gene of IBV consists of ORF1a and ORF1b, which is translated to 2 polypeptides, pp1a and pp1ab [5,6]. The replicase gene was elucidated to be a determinant of pathogenicity in IBV [7]. Four structural proteins are spike glycoprotein (S), small membrane protein (E), membrane glycoprotein (M), and nucleocapsid protein (N) [4]. The S glycoprotein is cleaved into S1 and S2 by cellular proteases and the S1 subunit produces serotype-specific and neutralizing antibodies [8-10]. Interactions between the membrane bilayer and the E and M proteins are important for virus budding and virus-like particles formation [11]. The 3a, 3b, 5a, and 5b accessory proteins are not essential to viral replication [12,13].

It is essential to know the antigenicity of the current IBVs in order to develop an effective vaccine, because the vaccine for IB prevention may not provide complete defense if the antigenicity is different. In Korea, IBV was first delineated in 1986 [14]. IB has been controlled by the use of live attenuated vaccine H120 strain and inactivated oil-emulsion vaccines containing both IBV KM91 and M41 strains [15]. Despite extensive vaccination, IB is still epidemic in Korea because of the frequent emergence of new variants [16-19]. Recombination and point mutation can lead to the emergence and evolution of new IBV variants [20]. Several studies on the molecular characterization of Korean IBVs have been reported [21-24]. However, most studies were mainly focused on partial sequences, including the S1 region. Because point mutation and recombination events were discovered to be distributed

throughout the whole genome, investigating only a small part of the genome may result in misleading conclusions [25-29]. Therefore, it is essential to define the extensive part of the genome. Up to now, comprehensive genetic data on IBV strains circulating in Korea has been very limited [16,18].

The purpose of this study was to determine the genomic sequences of Korean IBV isolates K046-12 and K047-12 and to investigate their potential genetic origin and variation, providing IBV molecular epidemiology information and the genetic characteristics of Korean IBV isolates for the prevention of IB.

Materials and Methods

Viral isolation and propagation

IBV K046-12 strain was isolated from the trachea of a 30-days-old broiler showing with inflammation in the trachea and ascites in broiler farm in 2012 in Cheorwon-gun, Gangwon province, Korea. IBV K047-12 strain was isolated from the cecal tonsil of a 6-weeks-old layer showing emaciation, inflammation in the trachea, thymic atrophy, renal enlargement and atrophy of systemic organs in Hy-line layer farm in

2012 in Pocheon-city, Gyeonggi province, Korea. These farms had a history of increased mortality. IBVs K046-12 and K047-12 were propagated in 9- to 11-day-old specific pathogen-free chicken embryos.

Primer design

Twenty-five primer pairs were made based on the published IBV genome sequence [28] and 2 primer pairs were designed using the primer walking method (Table 1). Eight other primers including K046-5'GSOP (TCTGCTCACTAACACCACC), K046-5'GSIP (CTACAAACGTCGTATAGCCG), K046-3'GSOP (CTCAGATGAGGAGAGGAACA), K046-3'GSIP (GGTGATTCTGCACTTGGTGA), K047-5'GSOP (CCTACAACCGTCGTATAGTC), K047-5'GSIP (ACCTTG-TGCGAGAACGTAGCGGA), K047-3'GSOP (GGGATTGACCT CAGATGAGGA), and K047-3'GSIP (GGGATTCAGCACTTGGAGAGA) were specifically made to amplify viral RNA 3'- and 5'-terminal end sequences.

Reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

For complete genome sequencing, viral RNAs were extracted

Table 1. Primers for the genome amplification and sequencing of infectious bronchitis virus K046-12 and K047-12 isolates

Primer pairs	Size	Upstream primer (5'-3')	Downstream primer (5'-3')
P1	1623	AGCGTTGGGCTACGTTCTC	CCAAATGCCCTCACCAAG
P2	801	GCGTACAATAGAGTGC GCAATC	GAGCAATTGTTCTACTGTGAGGTC
P3	1052	GGTTAGCATTGAGTGTGTGGTG	GAGACATATGCTCATTTGCGG
P4	1575	GGCATTGGATGAGTTAAAGAG	CGAACTTTAAGTGTCA GTGGCA
P5	1186	TGAAGTGGTAAAGGAAGATGTT	AAATCCTACACCAGTTTGCA
P6	1116	GGCAGGTTTTGTTATTATTG	AAATACTTAAAGACA ACTGTCAGACA
P7	1213	TTTTTGATAAATGCAGATGCT	TAGTAAATACAGTGGTTGCATAAG
P8	802	AATGGTTATAAAGTTTCAAGGTGT	CAACTTCAAATCATGATTATTAGC
P9	935	GCTGTTGAGAAGTGCATTGTT	ACACATCTACTCCAAAACCAAG
P10	1142	TAATCAGGTGGGTGGTGT	TCTTAAGTATATCATCACAATACTCAC
P11	1030	GCAAATTCAAAATGCATGCTTAT	CAAATTTACATGGTGGGTCTAAGTC
P12	1263	CAGCGTAGAGTCTAAATGTTATTA	GCCCATTTTAGCCAACAT
P13	821	AATAAGGATTGGTACGACCC	CCTTCCTTAAACATACCTGCTT
P14	1063	GAACTTGGTGTTATTATGAATCAAGATA	CAACATTAGCAGATGTGGCTTG
P15	1072	GTACTAGCAGTGATGCTAC	TCAACATTTTCGCTACCTACA
P16	845	TAGTATCTAATGGTACTGTTTTTGAATTT	TGGCAAGGCATTAATAGTACTAAAAAT
P17	858	AATTTCTTAAAGTTGATGATTGCACTC	TGCAAATTTTAAACAAACCTGTACCT
P18	1090	CGTGGTATACTTGTAGTCATGC	CTTTAGGGTTGCCTATATCATAG
P19	1181	CATATTGCAAATGAGGATGAAGT	ACATACAGATTGCTCCATCT
P20	792	GGTGTCTACCACGTGTATAAGC	GGCACACACATTGTAGTTTTAG
P21	773	CAAGTGGTATTATGATGAATGTGGC	AGTGACTTCCCCAACATCTCTAATC
P22	1118	AAGATGATAGTCCAGAAGAAT	AACACTATGCCATTAGGTGC
P23	748	TTGTGGTAGTGGAAGACATGT	CCATTAAACAGACTTTTTAGGTC
P24	1131	GAATCTTCTTATTACACTACTTTTGA	ACTCTCTATAGCAAAATTACATTGT
P25	1078	TTAGACTCTTAAAGCGGTGTAG	GAATACTAGGGAGGTACACGC
P15+	1072	GTACTAGCAGTGGTGTATGC	TCAACGTTTTTCGCTACCCACA
P25+	1141	GTAGGTCTTGGTGGTCATTTA	AGTCCGACTTTCAGATACTT

and purified with Viral Gen-spin Viral DNA/RNA Extraction kit (iNtRON, Korea) and stored at -70°C until further use. RT-PCRs were performed with a One-Step RT-PCR kit (Qia-gen, Germany) using respective primer pairs. Twenty-five overlapped polymerase chain reaction (PCR) fragments were amplified using specific primer pairs. The 5' and 3' end regions of the viral genomes were determined using the FirstChoice RLM-RACE kit (Ambion, USA) according to the manufacturer's instructions.

Cloning and sequencing

PCR products of the correct size were cut from agarose gel and purified using a MEGAquick-spin Fragment DNA Purification kit (iNtRON). The purified PCR products were then cloned into the pCR2.1-TOPO vector and transformed into the One-Shot TOP10 competent cells (Invitrogen, USA). Positive clones were extracted plasmid DNAs using DNA-spin Plasmid DNA Purification Kit (iNtRON). Confirmation of clones including recombinant plasmid was carried out by restriction enzyme digestion. Plasmid DNAs were sequenced in both directions by fluorescence-based sequencing (Macro-gen, Korea).

Sequence and phylogenetic analysis

The nearly complete genome sequences were assembled and analyzed using the Lasergene version 10 (DNASTAR, Inc., USA) computer program. These sequences were compared to each other and to the published IBV sequences by MegAlign Clustal W. Phylogenetic trees based on 1a, 1b, S, S1, S2, 3a, 3b, E, M, 5a, 5b, and N, and nearly complete genome sequences were generated by using the Lasergene software with bootstrap replicates for 1,000 replicates of original data. The sequence of IBV reference strains was retrieved from the GenBank database and accession numbers for the complete genomic sequences or nearly complete genomic sequences of 12 reference IBV strains and 2 reference human coronavirus strains used in this study are: H120 (FJ888351), IBVUkr27-11 (KJ135013), Arkansas vaccine (GQ504721), Connecticut vaccine (KF696629), Massachusetts (GQ504724), SNU8067 (JQ977697), KM91 (JQ977698), GX-C (KC008600), Sczy3 (JF732903), SDZB0808 (KF853202), CK,CH,IBTZ,2012 (KF663559), LX4 (AY338732), MERS-CoV-human-1 (KF958702), and TOR2-SARS CoV (AY274119).

Recombination analysis

Putative recombinant sequences were detected by the soft

Table 2. Nucleotide and amino acid identities of different regions between Korean IBV K046-12 and other IBV strain genomes

Strain	Complete genome	1a	1b	S	S1	S2	3a	3b	E	M	5a	5b	N
K047-12	89.0*	85.3 (87.1)	93.9 (97.1)	88.1 (87.8)	80.4 (78.1)	94.7 (96.2)	97.7 (100)	74.6 (65.1)	92.4 (89.1)	94.7 (96.0)	83.8 (83.3)	90.0 (85.5)	89.9 (94.1)
SNU8067	95.1	95.9 (96.6)	96.3 (97.7)	89.7 (88.5)	81.1 (78.0)	96.9 (97.0)	98.9 (98.3)	94.9 (93.8)	95.4 (93.6)	93.1 (96.9)	97.0 (98.5)	95.6 (90.4)	94.6 (96.8)
KM91	94.3	94.8 (95.6)	95.3 (97.0)	90.9 (90.0)	83.8 (82.3)	96.8 (96.7)	75.5 (61.2)	81.5 (74.6)	97.0 (96.4)	96.0 (98.2)	96.5 (98.5)	95.6 (91.6)	94.7 (95.6)
Sczy3	87.3	85.3 (87.1)	90.3 (95.8)	86.5 (87.4)	77.8 (77.7)	93.9 (95.6)	77.0 (69.0)	75.1 (65.1)	85.6 (83.5)	89.2 (93.4)	85.4 (80.3)	94.8 (89.2)	90.3 (93.9)
LX4	86.8	84.9 (86.5)	90.2 (95.7)	84.4 (85.4)	78.1 (76.2)	89.7 (93.0)	78.7 (70.7)	74.5 (65.6)	88.8 (84.5)	90.3 (91.6)	81.8 (74.2)	91.2 (91.6)	90.1 (93.7)
SDZB0808	87.2	85.2 (87.0)	90.2 (96.1)	86.3 (86.8)	77.6 (76.4)	93.7 (95.4)	78.2 (70.7)	75.1 (63.5)	85.6 (83.5)	89.7 (93.8)	83.8 (84.8)	92.0 (89.2)	90.9 (94.9)
CK, CH, IBTZ, 2012	85.3	85.5 (87.5)	89.9 (95.8)	70.7 (66.7)	65.1 (58.3)	75.6 (73.7)	79.3 (70.7)	82.5 (74.6)	90.1 (87.5)	88.3 (91.2)	81.3 (77.3)	92.0 (86.7)	90.7 (94.4)
GX-C	88.7	88.8 (90.3)	90.6 (95.5)	83.0 (83.6)	80.8 (78.1)	84.3 (87.8)	81.0 (79.3)	79.4 (68.3)	91.1 (89.9)	88.9 (91.2)	82.8 (80.3)	92.3 (87.8)	89.9 (92.7)
Connecticut vaccine	92.3	92.7 (93.6)	93.0 (97.0)	89.9 (90.2)	94.0 (90.7)	86.5 (89.7)	94.3 (93.1)	91.3 (89.2)	88.6 (84.3)	91.4 (93.3)	97.0 (97.0)	96.0 (92.8)	93.3 (95.6)
H120	91.5	91.0 (91.6)	93.1 (97.4)	91.2 (92.7)	98.4 (98.1)	85.4 (88.1)	81.0 (79.3)	85.1 (70.8)	86.7 (84.5)	88.6 (91.6)	93.4 (93.9)	94.0 (89.2)	90.6 (92.4)
IBVUkr27-11	90.1	90.7 (91.5)	91.9 (96.3)	84.1 (85.1)	83.2 (80.8)	85.2 (88.9)	89.1 (84.5)	91.3 (87.7)	88.1 (85.3)	89.7 (92.5)	86.9 (87.9)	95.6 (95.2)	91.2 (93.2)
Massachusetts	90.8	90.4 (91.9)	92.2 (96.6)	90.6 (91.7)	96.6 (95.7)	85.6 (88.3)	82.8 (77.6)	84.6 (70.8)	87.3 (87.3)	88.6 (92.0)	88.9 (86.4)	95.6 (92.8)	90.7 (93.9)
Arkansas vaccine	91.1	91.8 (92.2)	93.0 (96.5)	83.7 (83.5)	80.8 (76.9)	86.2 (88.9)	89.7 (86.2)	91.3 (87.7)	88.6 (85.2)	91.2 (93.3)	97.0 (97.0)	95.2 (90.4)	92.9 (94.9)

IBV, infectious bronchitis virus.

*Sequences with the higher or lowest identity with K046-12 at the nucleotide and amino acid level shown in bold. Parentheses indicate amino acid sequence identity.

package of Simplot (USA) [30]. The IBV nucleotide sequences from the nearly complete genome based on the multiple alignment data were introduced into similarity plots with SimPlot version 3.5.1. The nucleotide identity was calculated using the Kimura 2-parameter method with a transition-transversion ratio of 2 in each window of 200 bp, and the window was successively furthered along the alignment using a 20 bp increment.

Results

Sequence determination and analysis

The nucleotide sequence data determined in this study have been deposited in the GenBank database under the accession numbers MK618758 for K046-12 and MK618759 for K047-12. The nearly complete genome of K046-12 consists of 26760 nucleotides (nt). The nucleotide sequence length of the different ORFs 1a, 1b, S, 3a, 3b, E, M, 5a, 5b, and N were 11859, 7959, 3489, 174, 195, 333, 681, 198, 249, and 1230 nt long, respectively. The nearly complete genome of K047-12 consists of 27698 nt, including poly (A) tail and 5' (525 nt) and 3' (541 nt) UTRs. The nucleotide sequence

length of the different ORFs 1a, 1b, S, 3a, 3b, E, M, 5a, 5b, and N were 11856, 7959, 3489, 174, 189, 330, 681, 198, 249, and 1230 nt long, respectively. Sequence comparison analysis showed that the genomic nucleotide sequence identity between K046-12 and other IBV strains ranged from 85.3% (CK, CH, IBTZ, 2012) to 95.1% (SNU8067), and the genomic nucleotide sequence identity between K047-12 and other IBV strains ranged from 86.5% (IBVUkr27-11) to 94.8% (SDZB0808 [QX-like]).

The nucleotide sequence identity between the 1a, 1b, S, S1, S2, 3a, 3b, E, M, 5a, 5b, and N between K046-12 and other IBVs was 84.9% (LX4) to 95.9% (SNU8067), 89.9% (CK, CH, IBTZ, 2012) to 96.3% (SNU8067), 70.7% (CK, CH, IBTZ, 2012) to 91.2% (H120), 65.1% (CK, CH, IBTZ, 2012) to 98.4% (H120), 75.6% (CK, CH, IBTZ, 2012) to 96.9% (SNU8067), 75.5% (KM91) to 98.9% (SNU8067), 74.5% (LX4) to 94.9% (SNU8067), 85.6% (Sczy3 and SDZB0808) to 97.0% (KM91), 88.3% (CK, CH, IBTZ, 2012) to 96.0% (KM91), 81.3% (CK, CH, IBTZ, 2012) to 97.0% (SNU8067, Connecticut vaccine, and Arkansas vaccine), 90.0% (K047-12) to 96.0% (Connecticut vaccine), and 89.9% (K047-12 and GX-C) to 94.7% (KM91), respectively

Table 3. Nucleotide and amino acid identities of different regions between Korean IBV K047-12 and other IBV strain genomes

Strain	Complete genome	1a	1b	S	S1	S2	3a	3b	E	M	5a	5b	N
K046-12	89.0*	85.3 (87.1)	93.9 (97.1)	88.1 (87.8)	80.4 (78.1)	94.7 (96.2)	97.7 (100)	74.6 (65.1)	92.4 (89.1)	94.7 (96.0)	83.8 (83.3)	90.0 (85.5)	89.9 (94.1)
SNU8067	88.4	85.3 (87.4)	92.9 (97.2)	87.6 (87.6)	80.3 (77.8)	94.0 (95.9)	97.7 (98.3)	75.1 (65.1)	90.8 (88.1)	94.0 (93.8)	83.8 (83.3)	89.2 (85.5)	88.8 (93.7)
KM91	88.4	85.2 (87.0)	92.4 (96.8)	91.4 (92.0)	88.1 (87.1)	94.4 (96.2)	76.2 (61.2)	77.6 (67.7)	92.7 (91.8)	95.7 (97.8)	83.3 (81.8)	88.8 (84.3)	87.3 (92.0)
Sczy3	94.2	95.9 (96.1)	93.2 (97.1)	95.9 (96.2)	94.3 (93.7)	97.0 (98.1)	78.2 (69.0)	99.5 (98.4)	92.4 (92.7)	87.9 (90.3)	87.9 (87.9)	89.6 (85.5)	93.3 (95.9)
LX4	91.0	89.1 (89.4)	92.7 (96.6)	92.6 (93.1)	93.2 (91.2)	91.9 (94.5)	79.9 (70.7)	91.5 (84.1)	89.6 (87.2)	88.8 (88.5)	85.9 (83.3)	94.0 (92.8)	94.2 (96.3)
SDZB0808	94.8	95.8 (95.9)	94.8 (97.8)	96.1 (96.0)	94.8 (93.5)	97.0 (97.9)	79.3 (70.7)	98.4 (96.8)	92.4 (92.7)	87.9 (90.7)	95.5 (95.5)	96.4 (91.6)	93.7 (96.6)
CK, CH, IBTZ, 2012	91.1	96.1 (96.2)	93.3 (97.1)	71.1 (67.4)	65.7 (58.8)	75.9 (74.5)	78.7 (70.7)	80.3 (66.1)	85.1 (83.5)	88.8 (91.2)	85.4 (83.3)	89.2 (84.3)	94.7 (96.8)
GX-C	87.4	87.1 (88.7)	90.0 (95.7)	82.7 (83.6)	82.1 (79.9)	83.8 (86.5)	81.0 (79.3)	79.9 (67.7)	87.5 (87.2)	88.8 (88.9)	84.3 (83.3)	87.0 (81.7)	89.8 (93.2)
Connecticut vaccine	87.0	85.3 (87.3)	90.8 (96.2)	83.2 (83.9)	78.9 (76.8)	87.0 (89.9)	94.3 (93.1)	74.1 (63.5)	88.2 (86.0)	92.7 (93.3)	85.4 (83.3)	89.6 (86.7)	88.0 (92.4)
H120	87.2	84.9 (87.1)	91.3 (96.6)	83.0 (83.4)	79.5 (77.3)	86.3 (88.6)	81.6 (79.3)	79.4 (66.7)	87.8 (87.2)	88.8 (90.3)	87.9 (87.9)	88.8 (85.5)	88.0 (91.0)
IBVUkr27-11	86.5	84.9 (87.0)	90.3 (95.7)	82.6 (84.9)	79.2 (78.7)	85.7 (90.2)	87.9 (84.5)	74.6 (63.5)	88.3 (88.3)	89.9 (91.2)	88.4 (89.4)	89.2 (86.7)	87.0 (91.5)
Massachusetts	86.9	85.5 (87.2)	90.3 (95.8)	82.9 (83.0)	79.3 (76.8)	86.3 (88.4)	82.2 (77.6)	79.4 (68.3)	88.4 (89.9)	88.3 (90.7)	82.3 (78.8)	87.1 (80.7)	87.5 (91.0)
Arkansas vaccine	86.9	85.2 (87.1)	91.1 (96.2)	83.2 (84.0)	78.8 (77.2)	87.1 (89.9)	88.5 (86.2)	74.6 (61.9)	87.9 (85.0)	92.6 (93.3)	85.4 (83.3)	88.8 (84.3)	87.8 (92.2)

IBV, infectious bronchitis virus.

*Sequences with the higher or lowest identity with K046-12 at the nucleotide and amino acid level shown in bold. Parentheses indicate amino acid sequence identity.

(Table 2). The nucleotide sequence identity between the 1a, 1b, S, S1, S2, 3a, 3b, E, M, 5a, 5b, and N between K047-12 and other IBVs were 84.9% (H120 and IBVUkr27-11) to 96.1% (CK, CH, IBTZ, 2012), 90.0% (GX-C) to 94.8% (SDZB0808), 71.1% (CK, CH, IBTZ, 2012) to 96.1% (SDZB0808), 65.7% (CK, CH, IBTZ, 2012) to 94.8%

(SDZB0808), 75.9% (CK, CH, IBTZ, 2012) to 97.0% (Sczy3 and SDZB0808), 76.2% (KM91) to 97.7% (K046-12 and SNU8067), 74.1% (Connecticut vaccine) to 99.5% (Sczy3), 85.1% (CK, CH, IBTZ, 2012) to 92.7% (KM91), 87.9% (Sczy3 and SDZB 0808) to 95.7% (KM91), 82.3% (Massachusetts) to 95.5% (SDZB0808), 87.0% (GX-C) to 96.4%

(A)

(B)

(C)

(D)

(E)

(F)

Fig. 1. Phylogenetic trees generated by neighbor-joining method with 1,000 bootstrap replicates by means of Lasergene software based on complete and nearly-complete genome (A), and different regions of genome (B-M). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of each tree indicate the number of nucleotide substitution events. A dotted line on a tree indicates a negative branch length and bootstrap value > 50% are indicated for the major nodes. Putative recombinant IBV isolates (K046-12 and K047-12) are shown in bold. IBV, infectious bronchitis virus.

Fig. 1. continued

Fig. 2. Simplot analysis of IBV K046-12 (A) and K047-12 (B) isolates. IBV Massachusetts (green), SDZB0808 (yellow), and SNU8067 (blue) strains were used as putative parental strains when IBV K046-12 (A) and K047-12 (B) isolates were queried. The y-axis shows the percentage similarity within a sliding window of 200 bp centered on the position plotted, with a step size between plots of 20 bp. The breakpoint where the parental strains have equal identity to the query strain is the predicted recombination site. The genomic positions of the crossover sites were indicated by arrows with numbers. The genomic scale is shown at the bottom of the plot. IBV, infectious bronchitis virus.

(SDZB0808), and 87.0% (IBVUkr27-11) to 94.7% (CK, CH, IBTZ, 2012), respectively (Table 3).

Phylogenetic trees were made based on the nearly complete K046-12 and K047-12 genomes and the genomes of reference IBV strains. The nearly entire genome, 1a, 1b, S2, E, M, 5a, and N gene of K046-12 were closely related to Korean IBV isolates (SNU8067 and KM91). However, the S1 gene of the K046-12 was uniquely clustered with Mass-type IBV strains (H120, Massachusetts). The entire genome, 1a, 1b, S, S1, S2, 3b, E, 5a, 5b, and N gene of K047-12, was closely related to Chinese QX-like IBV strains (SDZB0808, Sczy3, and LX4). Interestingly, the 3a and M gene of the K047-12 is clustered with Korean IBV isolates (KM91, SNU8067, and K046-12) (Fig. 1).

Recombination analysis

Comparative analysis of the genomic SNU8067, SDZB0808, Massachusetts, and K046-12 sequences were performed, and the K046-12 sequence was used as a putative recombinant strain for the detection of the recombination events (Fig. 2). The results of recombination analysis showed that K046-12 was a recombinant strain with the major parental sequence came from KM91-like IBV (SNU8067) and minor parental sequence came from Mass-type IBV (Massachusetts) and QX-like IBV (SDZB0808). The regions of recombination were located at positions 15804-16765, 22904-23447 (higher identity with SDZB 0808), and 20192-21867 (higher identity with Massachusetts). Comparative analysis of the genomic SNU8067, SDZB0808, Massachusetts, and K047-12 sequences was performed, and the K047-12 sequence was used as a putative recombinant strain for the detection of the recombination events (Fig. 2). The results of recombination analysis showed that K047-12 was a recombinant strain with the major parental sequence came from QX-like IBV (SDZB0808) and minor parental sequence came from KM91-like IBV (SNU8067). Regions of recombination were located at positions 12717-13722, 17514-19926, 21772-22045, 23647-24063, and 24386-25388 (higher identity with SNU8067).

Discussion

IB is currently one of the most influential infectious diseases causing significant damage to poultry production [1]. New IBV variants have continuously emerged from point mutations and recombination. Although preventive vaccines have been used to prevent this disease, complete cross-protective immunity may not occur if the antigenicity of the currently prevalent virus and of the vaccine virus is different. Thus, for efficient control and prevention of IB, sequencing of the genome of the prevalent IBVs is needed to identify antigenicity and evolutionary patterns.

According to sequence comparison and phylogenetic analysis, K046-12 strain was most similar to Korean IBV strains (SNU8067 and KM91) and least similar to QX-type IBVs (Sczy3, SDZB0808, CK, CH, IBTZ, 2012, and LX4). How-

ever, the S1 gene of K046-12 had the highest nucleotide sequence identity with H120 strain at 98.4%. K047-12 strain was most similar to QX-like IBV strains (SDZB0808, Sczy3, CK, CH, IBTZ, 2012 and LX4) and least similar to Mass-type IBVs (Massachusetts, H120, and Arkansas vaccine). However, 3a and M genes were clustered away from other isolates of QX-like IBV (SDZB0808 strain) and were clustered with K046-12 and SNU8067 strains.

Recombination and point mutations are the main mechanisms of the emergence of new variant IBVs [27,29,31]. To verify the phylogenetic tree results, recombination analysis was performed. This study showed that recombination events occurred among traditional Korean IBV strains, QX-like IBV strains and vaccine strains. K046-12 may represent a recombinant strain derived from KM-like field IBV strain (SNU8067) and vaccine strain (H120) which has been extensively used as live vaccine. These results show that recombination of IBV can occur not only between currently prevalent IBVs, but also between current prevalent IBVs and IBVs used as live vaccines [17]. Also, the K047-12 may have been produced naturally by recombination resulting from co-circulation of KM-like field IBV strain (SNU8067) and QX-like field IBV strains. Presently, recombination 'hot spots' are described to be placed adjacent to the putative recombinant crossover sites, and these 'hot spots' are delineated to be A-T-rich motifs, e.g., CT(T/G)AACAA, CTT(A/T)(A/T)G [32]. In this study, A-T-rich motifs (CT(T/G)AACAA, CTT(A/T)(A/T)G) were discovered in regions adjacent to nearly all the putative crossover sites. However, it is hard to decide whether these motifs were associated with recombination or just appear around the recombinant sites at random [27,33]. Further analysis will be needed to find out whether these motifs are associated with the recombination [28].

The genetic and antigenic characteristics of IBV isolates have been studied principally based on the S1 gene, which is known to induce neutralizing antibodies [18,22,24,25]. However, such an analysis focused on the S1 gene alone may produce misleading results for IBV characteristics. In the case of K046-12, the 1ab genes, which are the key genes associated with pathogenicity, are closely related to KM91, but the S1 gene is closely related to Mass-type IBV. Therefore, it is necessary to analyze not only the S1 gene but also other genes including the 1ab gene in order to use the IBV isolate prevalent in a specific region as a vaccine [34].

In summary, this study shows that new emerging IBV isolates might have arose from recombination among co-circulating traditional Korean IBV strains, QX-like strains, and IBV vaccine strains. For effective IB control, it is necessary to continuously isolate and analyze the currently prevalent IBV, and include other genes, including the 1ab gene, as well as the S1 gene of IBV isolates. In addition to genetic analyses, the investigation of their antigenicity and pathogenicity, and cross-protection test by using *in vivo* experiment are needed for suitable vaccine development.

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