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ORIGINAL ARTICLE

Genetic and immunological characterization of commercial infectious bronchitis virus vaccines used in Korea

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Abstract
The aim of the study was to investigate the genetic and immunogenic features of commercial vaccines against infectious bronchitis virus (IBV), which is a major contagious pathogen of poultry. Although numerous vaccines have been developed based on the genetic characteristics of field strains, the continual emergence of variants decreases vaccine efficacy and cross-protection. To address this issue, we compared the S1 gene sequences of three IBV vaccines commercially available in Korea with those of various field isolates. Phylogenetic analysis showed that the vaccine strains clustered into two different lineages. Comparison of commercial vaccines with their parental viruses showed that most of the genetic variability occurred around hypervariable regions (HVRs). Conversely, antigenic stimulation with commercial vaccines and regional IBV variants was not sufficient to alter major immune cell phenotypes. Our study suggests that vaccines should be selected carefully based on their genetic background because genetic variability can affect the antigenicity of vaccines and host immune responses.

Introduction

Infectious bronchitis virus (IBV) is a coronavirus that was first isolated in the 1930s and has caused enormous economic damage globally. One of its four major structural proteins, the S glycoprotein, is important in several aspects of infection, including host invasion, host range, cell tropism, and induction of host immune responses. The S glycoprotein is synthesized as a precursor, which is cleaved to form the S1 and S2 subunits. The S1 subunit mediates host cell attachment, which initiates immune responses, and the S2 subunit bridges the viral membrane with the S1 subunit. Positive-sense single-stranded RNA viruses such as IBV have the highest mutation rates among all RNA viruses [1, 3, 8, 35]. Specifically, genetic mutation and recombination occur continuously in hypervariable regions (HVRs) of the IBV S1 glycoprotein because of the lack of a viral polymerase proofreading mechanism. This results in the continuous emergence of variants, which ultimately decreases vaccine efficacy and cross-protection [1, 3, 26, 35]. IBV variants are clustered into six genetically divergent groups and are further classified into 32 lineages, including genotype I (GI), with 27 lineages, and five other genotypes that are classified based on their S1 glycoprotein sequences [42].

Vaccination is an effective strategy for protection against IBV. The lifespan of broilers is short, and intensive vaccination is done at a very young age. Because of the heterogenicity of variants, IBV vaccines have been developed based on the antigenicity of regional isolates. The original IBV vaccine was derived from the Mass serotype, which belongs to the GI-1 lineage, and this has been a core component of the IBV vaccines. However, novel variants, such as nephropathogenic strains, exhibit more-complex pathogenesis. They initially invade the respiratory epithelium, penetrate deeper into the epithelial layer, and spread to remote organs, such as the kidney, liver, and spleen [34]. Most nephrotropic strains belong to the GI-19 lineage, which originated from the QX strain from China and QX-like strains that were further spread to European and Asian countries [20, 44, 45]. This has prompted a debate regarding the benefit of heterologous

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vs. homologous vaccination because of possible divergent immunity and the emergence of vaccine-escape variants [9]. Thus, the introduction of the protectotype concept, which involves combining two heterologous vaccine strains for an additive protection effect, has been suggested [14]. A critical step in vaccine development is the identification of field strains. Both inappropriate vaccine selection and low vaccine coverage in the field tend to increase the risk of generation of unexpected variants and reversion to virulence [10, 11]. After the initial IBV outbreak of 1986, live attenuated vaccines derived from the Mass strain (H120 and Ma5) were widely used in Korea up to the late 1990s. The subsequent emergence of nephrotropic strains prompted the development of the K2 vaccine strain, which is based on a Korean native strain, KM91. In the 2000s, a novel respiratory strain (KI strain) was detected in Korea, and other new variants were reported thereafter [7, 38]. We also analyzed the genetic features of the novel IBV strains K046-12 and K047-12, which were related to other Korean isolates and QX-like strains, respectively [25].

For better control of IBV using vaccination, we analyzed the genetic sequence of the S1 glycoprotein in three commercial vaccine strains. Using phylogenetic analysis, we examined the genetic relationship between vaccine strains and their parental strains and regional variants. The genetic stability of vaccine strains was evaluated by comparing their amino acid sequences. To evaluate IBV vaccine efficacy, recent studies examined the activity of immune cells, including T cell phenotype and function and antibody production, using animal infection models [32, 33]. Thus, the immune responses elicited by antigens from vaccine strains and regional variants were also examined.

Materials and methods

Vaccines and viruses

Three commercial live-attenuated IBV vaccines produced by different manufacturers were investigated. These included K40/09, Kr/D85/06, and a recombinant of the KM91 and QX IBV strains. Here, they were coded as A, B, and C, respectively, and their features and GenBank accession numbers are shown in Table 1. The lyophilized vaccines were reconstituted in PBS for sequence analysis as described previously [43]. In addition, vaccine B was propagated for one passage in 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Sungmin Farm, Korea).

PCR and cloning of the S1 gene

Total RNA was isolated from each vaccine using TRIzol Reagent (Invitrogen) and then reverse transcribed into cDNA using AccuPower RT PreMix (Bioneer) in an S1000 Thermal Cycler (Bio-Rad) according to the manufacturer's instructions. Each reaction was carried out in a final volume of 20 μl, and the resultant cDNA was used immediately for conventional PCR or stored at -70°C for later use. The S1 gene was amplified by conventional PCR using AccuPower PCR PreMix (Bioneer). The primer sets (forward: 5′-TAG TGA CCC TTT TGT GTG CAC TAT-3′ and reverse: 5′-GTT TGT ATG TAC TCA TCT GTA AC-3′) were used as described previously [22]. PCR was performed using a PCR Thermal Cycler Dice Touch (TaKaRa Bio Inc., Australia) with the following conditions: predenaturation at 94°C for 5 min; followed by 35 cycles of denaturation (94°C, 90 s), annealing (45°C, 30 s), and polymerization (72°C, 90 s); and a postpolymerization step at 72°C for 3 min. The amplified sequences were analyzed by 1% agarose gel electrophoresis. PCR products from the three vaccines and the single-passaged vaccine B were purified using a Gel Extraction Kit (QIAGEN), ligated into the TOPO TA pCR2.1 vector and introduced by transformation into One Shot TOP10 competent *E. coli* (Invitrogen) as recommended by the manufacturer. Transformants were grown in Luria-Bertani (LB) medium supplemented with kanamycin (50 ng/μl). Plasmid purification was performed using a DNA-spin Plasmid DNA Purification Kit (iNtRON), and the isolates were sequenced at Macrogen Inc. (Seoul, South Korea) using the universal primers M13F (5′-GTA AAA CGA CGG CCA GT-3′) and M13R-pUC (5′-CAG GAA ACA GCT ATG AC-3′).

Sequence comparisons and phylogenetic analysis

The sequences were analyzed using the Lasergene package version 10 (DNASTAR, Inc., USA) and deposited in the GenBank database under the following accession numbers: vaccine A, OM685193; vaccine B, OM685194; vaccine

Table 1 List of the commercial vaccine strains selected for this study. The parental strains, target, and GenBank accession numbers of each vaccine are indicated

Name	Parental strain	Target	GenBank ID
Vaccine A	K40/09	Respiratory and renal variants	OM685193
Vaccine B	D85/06	Respiratory and renal variants	OM685194
Vaccine B_single passage	D85/06	Respiratory and renal variants	OM685195
Vaccine C	KM91 & QX-like	Respiratory and renal variants	OM685196

B_single passage, OM685195; vaccine C, OM685196. The nucleotide sequences were aligned by the Clustal W method using the DNASTAR 2.0 MegAlign program, and a phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates using MEGA X software (version 10.2.2). For comparison, the nucleotide sequences of the S genes of the following strains were downloaded from the NCBI GenBank database: Conn48725 (FJ899692.1), Beaudette (M95169.1), M41 (AY561711.1), H120 (FJ888351.1), K046-12 (MK618758.1), BP-CaKII (MF924724.1), QXIBV (AF193423.1), K40/09 (HM486957.1), K047-12 (MK618759.1), ArkDPI (AF006624.1), GX2-98 (AY251816.1), K210-02 (AY257068.1), and Kr/D85/06 (EF621400.1). Chromatograms were reviewed manually using BioEdit version 7.2.5 to detect the presence of viral subpopulations in the vaccines.

Immune cell preparation and culture

Total splenocytes were collected from 4- to 5-week-old SPF chickens (Sungmin Farm, Korea). After RBS lysis, cells were seeded into 96-well U-bottom plates (5×10^5 cells/well) and cultured for 4-5 days with concanavalin A (5 μg/ml) (Sigma Aldrich, MO, USA) and chicken IL-2 (10 ng/ml) (Kingfisher Biotech, MN, USA) in RPMI-1640 supplemented with 10% FBS. For antigen stimulation, a suspension of vaccines and IBV variants (K046-12 and K047-12, 10 μL/mL) was added to the culture. The animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (no. KW-211021-6).

Cell staining and flow cytometry

Cultured splenocytes were harvested and stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain (L3457; Thermo Fisher) and antibodies specific for CD4 (CT-4, SouthernBiotech), CD8α (CT-8, SouthernBiotech), CD44 (AV6, SouthernBiotech), monocyte/macrophage (KUK01, SouthernBiotech), MHCII (2G11, SouthernBiotech), and Bu-1 (AV20, SouthernBiotech). Stained cells were analyzed using flow cytometry (Cytoflex, Beckman Coulter).

Statistics

Dunnett's test with ANOVA was used to assess the statistical significance of the results obtained from the viral-antigen-stimulated groups compared with the untreated group. Comparisons among the stimulated groups were analyzed by one-way ANOVA. Significance was set as $P < 0.05$.

Results

Cloning of the S1 glycoprotein gene from commercial vaccine strains

The PCR products of the S1 glycoprotein gene from three different commercial vaccine strains and the single-passaged commercial vaccine strain were inserted into the pCR2.1 vector, which contains a kanamycin resistance gene (Fig. 1A). The inserted PCR product (~1,400 bp) was verified by digestion of cloned plasmids with EcoRI, followed by gel electrophoresis (Fig. 1B). This procedure confirmed

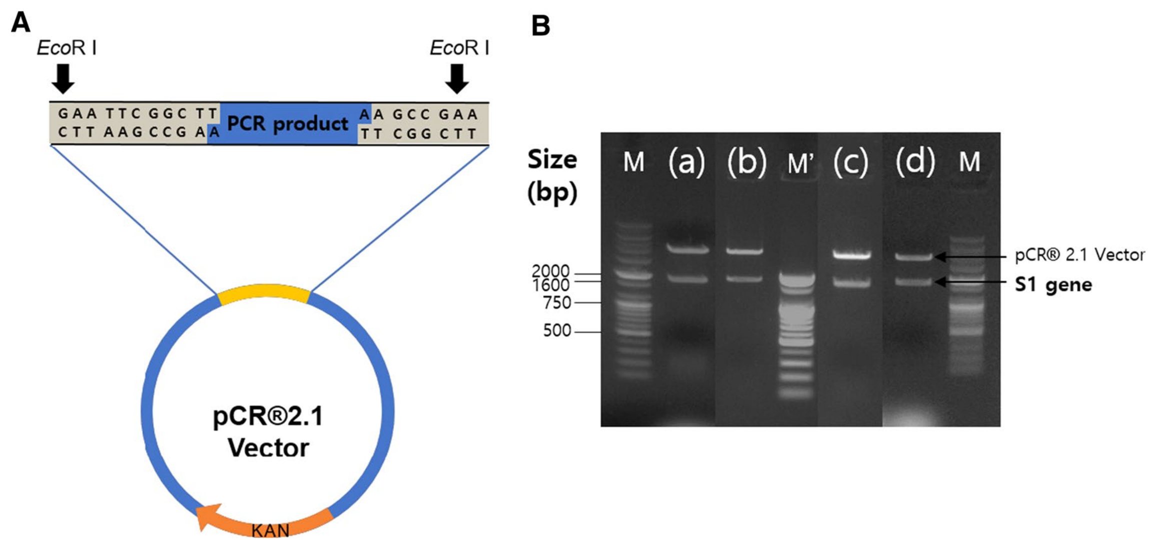


Fig. 1 Cloning of the S1 glycoprotein gene from commercial vaccines. (A) The S1 gene was inserted into the pCR2.1 vector using EcoRI restriction sites. (B) The inserted PCR product was verified using the EcoRI restriction enzyme. The four lower bands correspond to the inserted S1 glycoprotein in vaccine strains, and the upper bands are the cloning vectors.

the suitability of the preparation for sequencing of the S1 gene.

Classification of vaccine strains and IBV variants based on S1 glycoprotein gene

We used the sequences of the full-length S1 genes of commercial vaccines (Supplementary Fig. S1) to investigate their genetic relatedness to their parental strains and 10 different field strains of five distinct lineages. In addition, two Korean isolates (K046-12 and K047-12; the genetic features of which were reported by us in 2019) [25] were also included. The S1 sequences of vaccines A and C were grouped in the GI-19 lineage, which includes nephrotropic strains such as the QX strain and its regional variant K047-12. As expected, vaccine A was genetically close to its parental strain K40/09 and its passaged strain K40/09 CE50. In contrast, vaccine C was genetically more closely related to the K40/09 strain than to the KM91 and QX-like strains. Vaccine B and its single-passaged form grouped in the GI-15 lineage, together with their parental strain, D85/06. Otherwise, none of the commercial vaccine strains belonged to the conventional respiratory GI-1 lineage, which includes the Connecticut, Beaudette, M41, and H120 strains (Fig. 2). Although most of the recent vaccines target nephrotropic strains that induce a complex pathology [34], the recent variant K046-12 was genetically remote from the three commercial vaccine strains. This phylogenetic analysis indicated that the S1 genes of commercial IBV vaccine strains cluster according to their parental strains but do not completely cover emerging variants.

Amino acid sequence variation in the S1 glycoproteins of vaccine and parental strains

The S1 glycoprotein consists of 520 amino acid residues and induces a neutralizing antibody response. Frequent genetic modifications in the HVRs prevent viral clearance from the host. The major HVRs (HVRI, II, and III) of the S1 glycoprotein include amino acid residues 38-67, 91-141, and 274-387, respectively [4, 26]. A comparison of the amino acid sequences of the HVRs and HVR-adjacent regions showed that vaccine A, which originated from a nephrotropic virus, showed sequence variation within its HVRs when compared with the parental strain K40/09 and its passaged strain, K40/09 CE50. The amino acid at position 83 is an asparagine (Asn) in the K40/09 strains, whereas it is an aspartic acid (Asp) in the vaccine A strain. Similarly, the amino acids at positions 97 and 365 are serine (Ser) and isoleucine (Ile) in the K40/09 strains, whereas they are phenylalanine

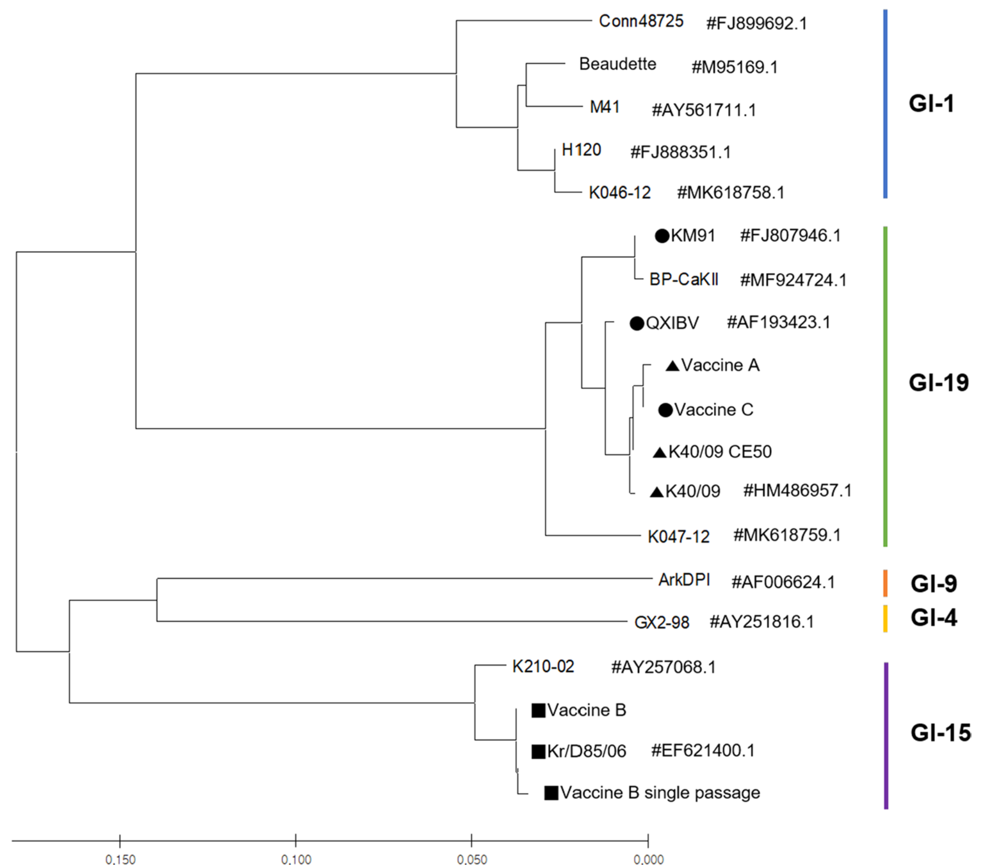


Fig. 2 Phylogenetic analysis of the S1 gene sequences of vaccine strains and variants. Vaccine A and its parent strain are indicated by triangles (▲), vaccine B and its parent strain are indicated by squares (■), and vaccine C and its parent strains are indicated by circles (●). The five different lineages of the strains are indicated in bold. The tree was created using the neighbor-joining method in MEGA, version 10.2.2, with 1000 bootstrap replicates.

(Phe) and valine (Val) in the vaccine A strain. Conversely in the HVRIII-adjacent area (412 amino acid residues), all three vaccine strains have a glutamic acid (Glu), with a point mutation detected only in vaccine A. This indicates that the difference from K40/09 was more pronounced in vaccine A than in strains that had undergone 50 passages (Fig. 3A). Amino acid sequence variability in vaccine B was mostly observed adjacent to HVRI at positions 8, 11, and 13, compared with its parental strain D85/06. AVR1/08 is an attenuated vaccine strain that was passaged 89 times [7], and it showed more genetic similarity upstream of the S1 gene to D85/06 than to vaccine B. The amino acids encoded were valine (Val), isoleucine (Ile), and phenylalanine (Phe) for D85/06 and AVR1/08, whereas the vaccine B strains carried leucine (Leu) or cysteine (Cys). Moreover, at the position 56, D85/06 encoded serine (Ser) but other strains encoded tyrosine (Tyr). In addition, a single passage of vaccine B yielded a change from glutamic acid (Glu) to glycine (Gly) in HVRII (amino acid residue number 96) (Fig. 3B). Despite the overall similarity in the whole S1 sequence, the vaccine strains under study contained multiple polymorphisms in the HVRs and upstream of the S1 gene.

Genetic variation in the S1 glycoprotein among commercial vaccines and new variants

Next, we addressed the genetic similarity of the S1 gene among the three commercial vaccine strains. Dissimilarity was detected preceding HVRI and HVRII at the amino acid positions 83, 257, and 262. Although vaccines A and C were genetically closer in terms of the whole S1 protein sequence (Fig. 2), vaccines A and B exhibited an identical amino acid sequence near HVRI and II (Fig. 4A). Furthermore, at amino acid position 315, vaccine C showed a secondary peak in the electropherogram that encoded serine (Ser), whereas the major peak of each of three strains encoded cysteine (Cys). This suggests the existence of two viral subpopulations in the vaccine (Fig. 4B). Because the three vaccines are designed to deal with regional variants, we examined whether these vaccines are genetically close to emerging Korean variants, the genetic features of which were previously analyzed by us [25]. The amino acid sequences of HVRI (up to position130) from K046-12 and K047-12 were aligned with those of the three commercial vaccine strains. Heterogeneity was most frequently observed around the amino acid positions 30-50 and 120-130 (Fig. 5A). To further analyze the genetic relationships of the HVRI sequence, we constructed another phylogenetic tree based on the deduced amino acid sequence of this region. We found that vaccines A and C were grouped with the nephrotropic variant K047-12, whereas vaccine B was less closely related. Moreover, none of the vaccine strains were closely related to the respiratory variant K046-12 (Fig. 5B). These results agree with those obtained using whole S1 gene sequences, which implies that HVRI variation is an important factor determining the genetic characteristics of both field and vaccine strains.

Immune responses to commercial vaccines and regional variants

We recently reported the action of the nephrotropic variant K047-12 on the innate immune response and identified key immunological factors that govern the acute response

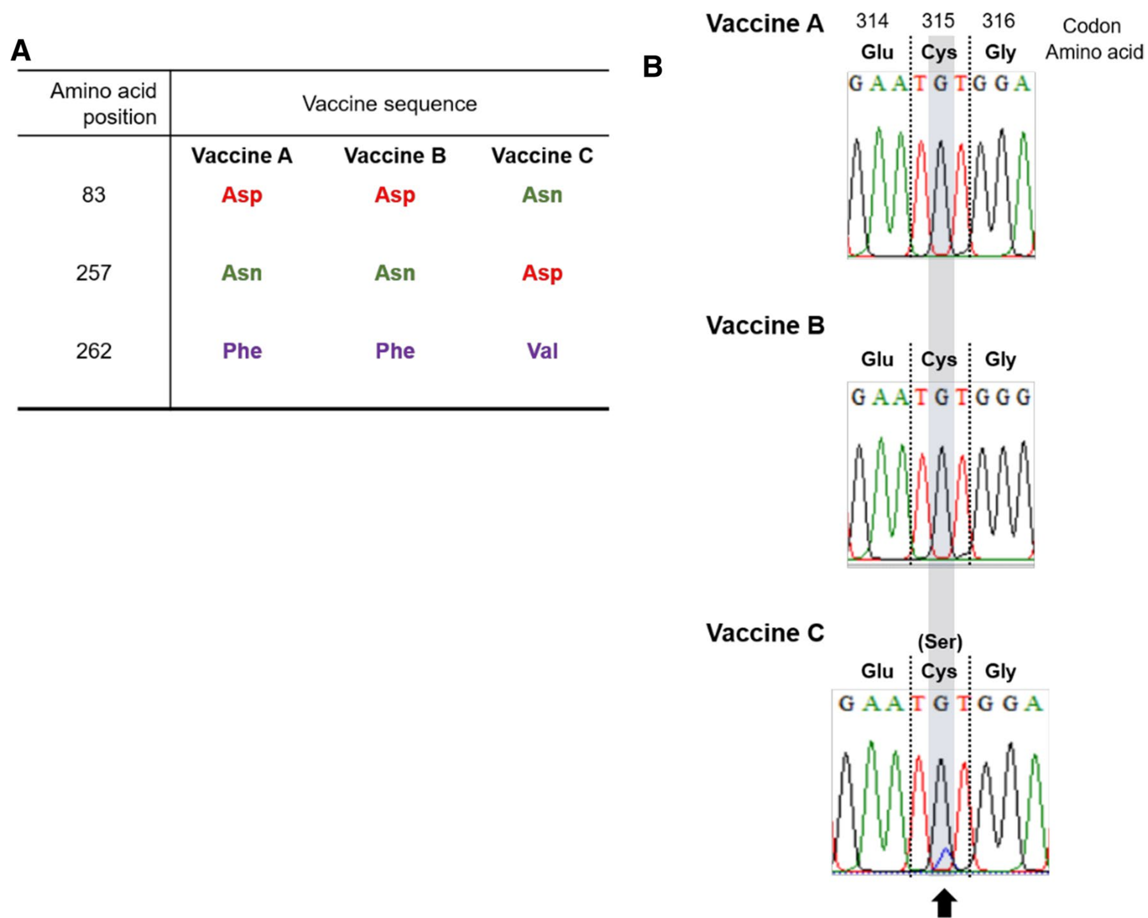
A

Amino acid position	Vaccine A sequence		
	K40/09	K40/09 CE50	Vaccine A
37	Ser AGT	Arg AGG	Arg AGG
83	Asn AAC	Asn AAC	Asp GAC
97	Ser TCC	Ser TCC	Phe TTC
365	Ile ATT	Ile ATT	Val GTT
412	Glu GAA	Glu GAA	Glu GAG

B

Amino acid position	Vaccine B sequence			
	D85/06	AVR1/08	Vaccine B	Vaccine B single passage
8	Val	Val	Leu	Leu
11	Ile	Ile	Leu	Leu
13	Phe	Phe	Cys	Cys
56	Ser	Tyr	Tyr	Tyr
96	Glu	Glu	Glu	Gly

Fig. 3 Genetic divergence of commercial vaccines from parental strains. The amino acids encoded by the S1 gene were compared at multiple sites. (A) Amino acid sequence differences between vaccine A from K90/09 and K40/09 CE50. (B) AVR1/08 and D85/06 were compared with vaccine B before and after a single passage. The properties of the amino acids are represented as follows: purple, nonpolar; green, neutral polar; red, acidic polar; and blue, basic polar.



Amino acid position	Vaccine sequence		
	Vaccine A	Vaccine B	Vaccine C
83	Asp	Asp	Asn
257	Asn	Asn	Asp
262	Phe	Phe	Val

Fig. 4 Amino acid sequence variation among vaccine strains. (A) Amino acid sequence differences among vaccines A, B, and C. (B) Chromatogram showing heterogenicity in the S1 gene. The secondary nucleotide peak position encoding at the amino acid residue 315 is indicated by an arrowhead. The properties of the amino acids are represented as follows: purple, non-polar; green, neutral polar; red, acidic polar; and blue, basic polar.

in chicken embryonic kidney (CEK) cells [19]. The avian spleen, similar to that of mammals, is a major secondary lymphoid tissue in which various immune cells, such as lymphocytes and myeloid cells, regulate adaptive immunity. In the spleen, dendritic cells and macrophages recognize antigens and present them to lymphocytes, i.e., T and B cells. Activated lymphocytes differentiate into effector cells, thus initiating both cellular and humoral immune responses [12, 40]. Avian immune cells can be characterized by flow cytometry, which enables both quantitative and qualitative analysis of lymphocytes [13, 36]. We examined the manner in which viral antigens in vaccine strains and regional variants affect the immune cell phenotype. Total splenocytes from 4- to 5-week-old SPF chickens were stimulated in the presence of the three vaccine strains or the regional variants (K046-12 and K047-12). To examine the memory T cell population, which potentially interacts with viral antigens, we stained the surface of $CD4^+$ and $CD8^+$ T cells with an anti-CD44 antibody. None of the treatments changed the frequencies of memory $CD4^+$ and $CD8^+$ T cells (Fig. 6A). To assess the antigen-presenting capacity of macrophages, monocytes were stained with an antibody to the MHCII molecule. Although slight activation of macrophages by IBV was observed, the differences between the variant and vaccine strains were not significant. A similar trend was observed for B cell frequency (Fig. 6B, C). These data suggest that antigenic stimulation *in vitro* is not sufficient to change the surface phenotype of major immune cells. Further functional analysis of intracellular molecules and cytokine regulation within each cell type is needed to evaluate these issues.

Discussion

As novel nephropathogenic IBV serotypes are isolated, numerous IBV vaccines are introduced. This emphasizes the importance of proper vaccine selection against regional

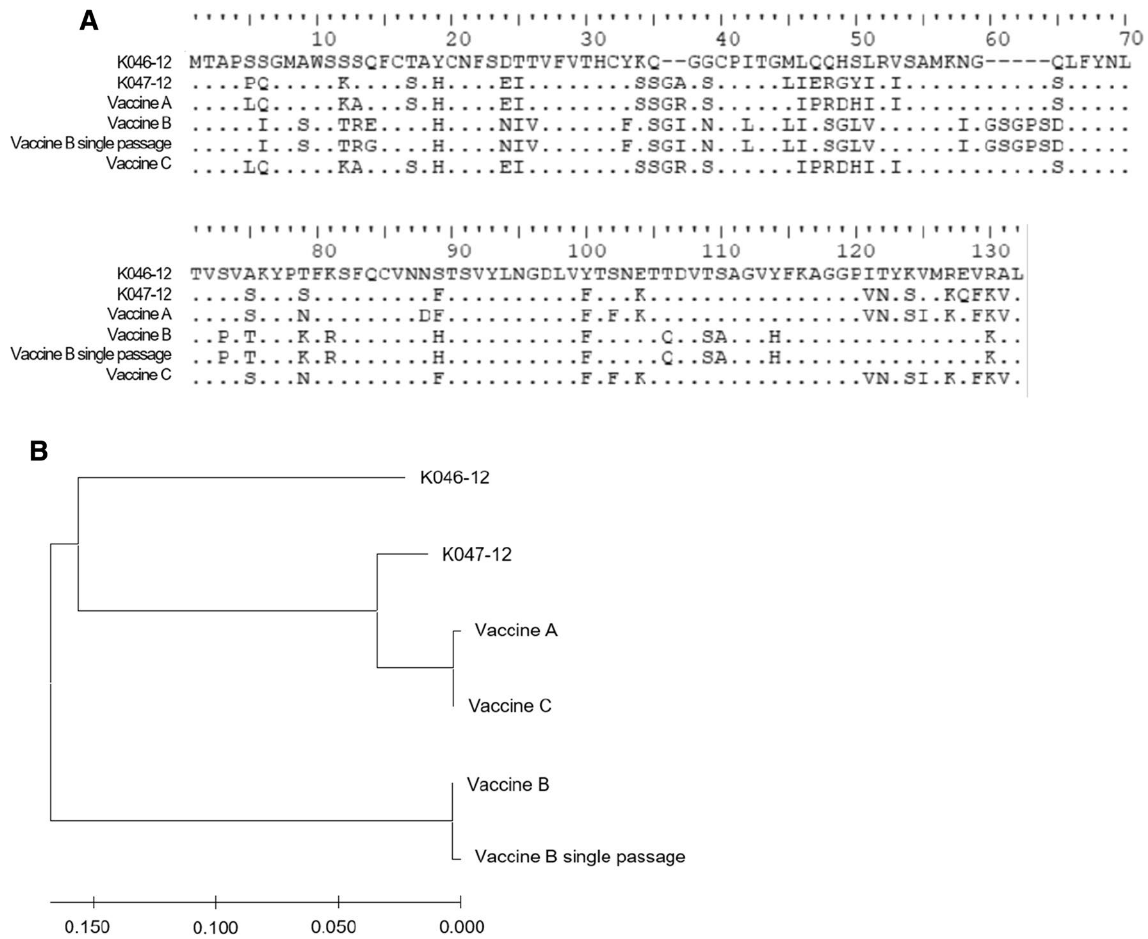


Fig. 5 Genetic similarity of HVRs among the three vaccine strains and the regional variants. (A) Alignment of the amino acid sequences encoded upstream of the S1 gene. (B) Phylogenetic analysis based on the amino acid sequence of HVRI.

variants [15]. To better evaluate vaccine stability and antigenicity, we determined the S1 sequences of three commercial vaccines and of various field strains. The genetic variation of the three vaccine strains was assessed by comparing them with the parental strains. The vaccine strains exhibited genetic modification primarily in HVRI, HVRII, and their adjacent regions However, compared with the regional variants, antigenic stimulation with the vaccine strains did not significantly change the phenotype of major immune cells.

The majority of emerging IBVs are classified as GI-19, and we observed genetic distance between vaccines A and C and other field strains. The parental strain of vaccine A, K40/09, was developed from a QX-like strain that was reported in 2011 and is thought to have been generated by recombination with a KM91-like strain [21]. When chickens were immunized with K40/09, cross-protection against both respiratory and renal IBV strains was observed [16]. Vaccine A maintained genetic stability compared with its parental strains, and it was also genetically close to the new regional variant K047-12. HVRI includes the receptor-binding domain, which determines the interaction between the virus and respiratory tissues. Hence, genetic modification of amino acid residues in this region can generate novel variants [31, 35]. The HVRI amino acid sequences differed between K40/09 and vaccine A to a certain extent, although less genetic variation in HVRI was observed after 50 passages. Therefore, whether vaccine A is sufficient to provide broad immunity to novel variants warrants further exploration. Moreover, the potential risk of recombination with field viruses should be evaluated.

KM91 is a native Korean variant that was first isolated in 1991, and KM-like variants have been isolated that exhibit evidence of recombination with QX-like strains [6, 19]. According to the manufacturer, vaccine C is a recombinant of the QX and KM91 strains that targets both respiratory and nephropathogenic IBV; however, it is difficult to determine the parental strain in this case. Our analysis showed that vaccine C also belonged to the GI-19 lineage, which confirmed that this strain stems from the KM91 and QX-like strains. Considering the genetic analysis of the S1 glycoprotein and

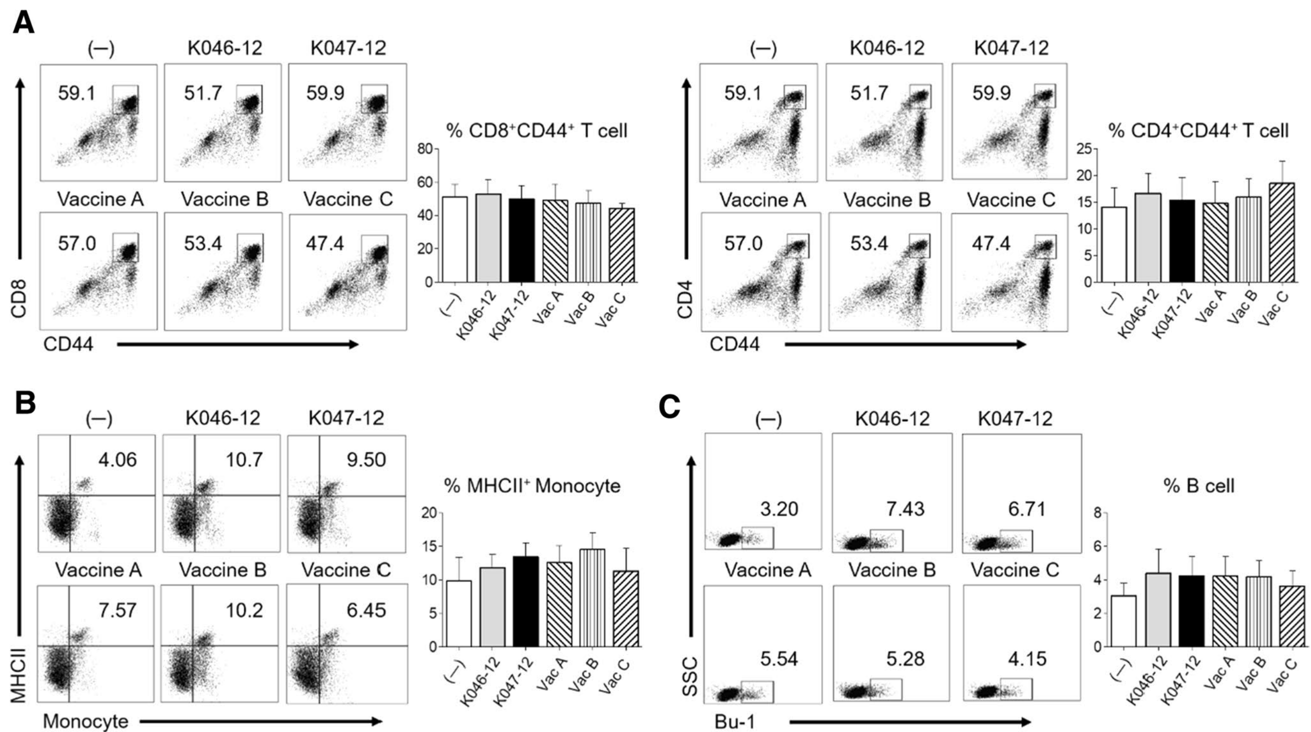


Fig. 6 Immune cell phenotypic changes induced by viral antigen stimulation. The frequencies of memory $CD4^+$ and $CD8^+$ T cells (A), $MHCII^+$ monocytes (B), and B cells (C) are shown. Total splenocytes from SPF chickens were stimulated in the presence or absence of viral antigens from vaccine or variant strains. Representative and pooled data were obtained (mean ± SEM) from four independent experiments. "*" indicates a significant difference between the indicated groups ($P < 0.05$).

the information provided by the manufacturer, we suggest that vaccine C shares a genetic background with vaccine A. However, the efficacy of the vaccines against new regional respiratory variants such as K046-12, which belongs to the GI-1 lineage, remains to be examined.

The D85/06 strain was isolated in Korea in 2006 and clusters in the K-I type together with other respiratory IBV strains. The AVR1/08 strain was derived from the D85/06 strain via 89 passages. After 47 passages, point mutation was observed at amino acid position 56 of AVR1/08 that attenuated the virulence of the strain [5, 18]. Vaccine B was introduced from AVR1/08 and showed genetic modification upstream of HVRI. We performed a single passage of vaccine B to evaluate its genetic stability and detected a point mutation in HVRI after this procedure. According to a previous report, AVR1/08 yielded broad protection against both regional respiratory and renal IBVs [7]. However, our phylogenetic analysis revealed that vaccine B was grouped into a genetically separate lineage. Moreover, vaccine B was genetically distant from respiratory strains such as M41 and Beaudette and the regional isolate K046-12. A comparison of the three vaccine strains revealed a genetic difference outside the HVRs. To estimate the protective efficacy of vaccines against novel IBV strains, we aligned the HVRI amino acid sequences of the three commercial vaccines with those of the regional isolates K046-12 and K047-12. A major difference was observed upstream of HVRI. Of note, the phylogenetic distance based on HVRI was comparable to that obtained using the whole S1 gene sequence. Our results suggest that the protectotype concept is applicable to the three vaccine strains, whereas an alternative vaccine for respiratory strains is necessary to provide broader protection against emerging variants.

The induction of adaptive immunity is accelerated when an IBV-specific epitope stimulates a cytotoxic T lymphocyte (CTL) response. An S1-glycoprotein-associated peptide can function as an epitope. When chicken splenocytes were activated with a peptide epitope, IFN-γ production and $CD8^+$ T cell proliferation were facilitated. Furthermore, administration of this epitope induced a DNA-vaccine-like effect [37, 41]. The major purpose of vaccination is to induce specific immune responses against an invading pathogen. However, IBV variants are likely to escape host immune surveillance by modifying their antigenicity because genetic changes in the S1 glycoprotein alter the affinity of the virus for host cell receptors. As shown previously, protection can be achieved by adoptive transfer of memory $CD8^+$ T cells. The key protective action was shown to be mediated by memory T cells that recognized the IBV antigen [29]. IBV variants with dissimilar amino acid residues in the S1 glycoprotein are less

likely to express a coherent epitope. However, we found that the antigenic variation in S1 was not sufficient to alter the immune cell phenotype. This implies that an immune-boosting strategy needs to be considered during vaccine development. Antigen presentation and cytokine production by macrophages are required for the induction of pathogen-specific T cell activity. For example, IFN-γ production in CTLs is mediated by M1 macrophage generation during intracellular pathogen infection [27]. The role of macrophages in IBV infection is also crucial. Upon infection with the M41 variant, the viability and phagocytic function of macrophages are inhibited. Conversely, aspects of overall innate immunity were enhanced, such as antimicrobial activity, Toll-like receptor (TLR) activation, and type I IFN or pro-inflammatory cytokine induction [39]. We detected a small number of activated macrophages that expressed MHCII molecules on their surface, probably because the inducing conditions for myeloid cells were not optimal. Therefore, a comprehensive analysis of viral-antigen-induced macrophage activity needs to be carried out using bone-marrow-derived myeloid cells and stimulating cytokines. Moreover, revised IBV vaccines that boost MHCII expression on myeloid cells for enhanced antigen presentation can be applied [17]. This will provide improved antigen-specific T cell proliferation and afford immunity geared toward novel variants. In addition to cellular immunity, a humoral response by B cells is required. IBV-specific antibody-secreting cells (ASCs) are maximally activated around 10 days after IBV infection. However, during *in vitro* culture, splenic ASCs are transiently observed only when restimulated with the viral antigen [30]. In our splenocyte culture system, viral antigens enhanced B cell frequency, but the level of the response did not depend on the antigens. Although we did not include *in vivo* models, future studies should be planned to evaluate the efficacy of commercial vaccines against regional variants. For deeper understanding of host immune responses to viral antigens, animal infection models can be conducted in the future, according to the terrestrial manual of International Office of Epizootics (OIE). For example, the rate of virus detection in the vaccinated group should be lower than 20%, while in the control group, it should be higher than 80%. This is used as a standard for vaccine evaluation [2, 23, 24]. In addition, both humoral and cellular immune responses boosted by vaccination, such as the neutralizing titer, the number of IFN-γ producing cells, and proliferation of $CD4^+$ and $CD8^+$ T cells, should be evaluated. Histopathology and viral load in target organs, survival rate, and tracheal ciliary activity can also provide more information for vaccine-mediated immune responses [28, 32, 33].

The inconsistent antigenic phenotype of IBV variants is a major hurdle for IB prevention, despite the continuous production of vaccines. The majority of IBV vaccines are of the live-attenuated type, which requires stability during the manufacturing process. In the host, vaccines should be safe and provide long-lasting immunity to multiple variants. In this study, we investigated the genetic features and stability of IBV vaccines that are currently available in Korea. In addition, the possible host immune response was examined after the stimulation of host cells with the viral antigen. Our study provides guidance for the evaluation and updating of commercial vaccines.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-022-05519-2.

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