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Differential evolution of antigenic regions of porcine reproductive and respiratory syndrome virus 1 before and after vaccine introduction

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is a widespread viral pathogen that has caused tremendous economic losses throughout most pig-producing countries. Nowadays, both PRRSV-1 and PRRSV-2 co-circulate in Korean pig populations, and commercial modified live vaccine (MLV) is predominantly used to control PRRS. Specifically, control strategy using only PRRSV-2 MLV that was used since 1995 cannot prevent the spread of PRRSV-1 and damage from its infection, which led to the first introduction of two additional PRRSV-1 vaccines in 2014. Despite the wide implementation with PRRSV-1 vaccines, there is a lack of knowledge about the currently circulating Korean PRRSV-1 strains. Whole structural genes of PRRSV-1 before (11) and after (17) the introduction of vaccine were compared to determine the genetic evolutionary features of PRRSV. Genetic and phylogenetic analysis indicated that Korean PRRSV-1 shared 91.5 ± 1.7% nucleotide identity but formed a unique clade based on ORF2–7 phylogeny. Bioinformatics showed increased genetic heterogeneity, enhanced diversifying selection, and the emergence of novel glycosylation sites within neutralizing epitopes of minor structural proteins after vaccine introduction. Taken together, our data provide novel insight into the evolution of minor structural proteins of PRRSV-1 in the vaccination era.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus that belongs to the *Arteriviridae* family within the order *Nidovirales*. Its genome consists of single-stranded, positive-sense RNA that is 15–15.5 kb in length and encodes 10 open reading frames (ORF). Two large ORFs, ORF1a and ORF1b, occupy three-quarters of the genome and produce two polyproteins that are cleaved into 14 nonstructural proteins (nsp). Nsp is involved in viral transcription and translation, as well as in the suppression of innate immunity through their interference with various signaling pathways (Han and Yoo, 2014). In the 3′ terminal region, structural genes (ORF2a, ORF2b, ORF3–7, and ORF5a) comprise the remaining one-quarter of the genome, encoding glycoprotein 2 (GP2), small envelop (E), GP3, GP4, GP5, membrane (M) protein, nucleocapsid (N) protein, and ORF5a protein (Johnson et al., 2011; Meulenberg et al., 1993; Wu et al., 2001). As the most abundant structural protein, N protein is the sole structural component of the viral capsid, which interacts with viral RNA and is highly immunogenic in pigs (Snijder and Meulenberg, 1998). The viral capsid is encapsulated by a lipid envelope containing the GPs and M protein. GP5 and M protein form a disulfide-linked heterodimer that is essential for viral assembly, and which interacts with cellular receptors (Delputte et al., 2002; Verheije et al., 2002). GP2a, GP3, and GP4 are considered to be present at low levels on the envelope of the PRRSV virion.

There have been many studies in which ORF5 and/or ORF7 were predominantly used to characterize the epidemiology and evolution of PRRSV due to large quantities of available sequence data (Shi et al., 2010a,b; Stadejek et al., 2008). The emphasis on ORF5 was initially attributed to its significant role in viral pathogenesis and immunity and its genetic characteristics, as it shows the highest variability among structural proteins. However, the continuous evolution of PRRSV over the past 30 years has resulted in the genetic heterogeneity of various regions of the PRRSV genome, which suggests that short sequences might provide only limited information when establishing phylogenetic relationships and determining evolutionary features of PRRSV. In

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addition, recent studies have provided critical clues regarding the function of minor structural proteins in PRRSV pathogenesis (Das et al., 2010; Tian et al., 2012). For PRRSV-1, the elucidated importance of minor structural proteins has continuously increased with the discovery of neutralizing epitopes in GP2a, GP3, and GP4, but not in GP5 (Vanhee et al., 2011).

PRRSV-2 has been widely distributed in most-pig-producing countries and novel highly pathogenic variants continuously emerged (Bush et al., 1999; Han et al., 2006; Tian et al., 2007). In contrast, the distribution of PRRSV-1 has been restricted including Europe, USA, Canada, South Korea, China and Thailand, which results in relatively less available data about genetic information (Shi et al., 2010a). Hence, our group characterized the ORF4 to ORF6 of Korean PRRSV-1 field strains which circulated in 2012. We noted a high level of diversity among Korean PRRSV-1 and observed that ORF4 is remarkably variable among field strains (Lee et al., 2017). Since the first identification of PRRSV-1 and PRRV-2 in 2005 and 1994, respectively, both viruses co-circulated in Korean pig populations (Kim et al., 2006; Kweon et al., 1994). PRRSV-2 modified live vaccines (MLV) that was licensed in 1995 had been a sole option in order to control PRRSV before 2014. Despite the extensive implementation of PRRSV-2 MLV in the field, PRRSV-1 had become increasingly prevalent and continuously caused enormous economic burdens. These circumstances led to the first introduction of two additional PRRSV-1 MLV (Porcilis PRRS and Unistrain PRRS) in 2014. However, vaccination plays a critical role in viral evolution, increasing the evolutionary rate, affecting positive selection, and resulting in the emergence of escape variants (Costers et al., 2010b; Lee et al., 2016; Mortara et al., 1998; Park et al., 2011). Because commercial vaccines against this virus fail to confer sterilizing immunity against field strains, PRRSV might frequently mutate to adapt to new immune environments of the host animals. Unfortunately, there has been little information regarding Korean PRRSV-1, which was identified after the introduction of the vaccine. Therefore, since we had relatively large number of the field samples collected before vaccine introduction, this study aimed to investigate the genetic and evolutionary characteristics of whole structural genes of Korean PRRSV-1 before and after the introduction of vaccines in the field.

2. Methods

2.1. RT-PCR and sequencing analysis

KU viruses comprised 11 (before vaccine introduction) and 16 (after vaccine introduction) viruses that were identified between 2012 and 2013 and between 2015 and 2017, respectively. The 27 PRRSV-1-positive samples originated from different regions and farms. Total RNA was extracted from clinical samples using Qiazol Lysis Reagent (Qiagen, MD, USA) according to the manufacturer's instructions. RNA was mixed with PRRSV-specific primers to synthesize cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Whole structural genes were amplified using two sets of PRRSV-1-specific primers that produced two overlapping amplicons. The primers used in this study are listed in Table 1. The PCR reaction was carried out using Takara Ex Taq (TaKaRa Bio, Shiga, Japan) using the following conditions: 35 cycles of 10 s at 98 °C, 30 s at 53 °C, and 2 min at 72 °C. The PCR products were separated by gel electrophoresis and purified using a commercial gel extraction kit (DokDo-Prep™ Gel Extraction spin-type Kit, ELPIS BIOTECH Inc., Daejeon, Korea). Purified DNA was sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea) using the same primer sets. The sequences of ORF2–7 of 27 KU viruses used in this study were deposited in GenBank under accession numbers KY996316–KY996336 and MG999907–MG999912.

2.2. Phylogenetic analysis

PRRSV-1 reference sequences that were previously deposited in the GenBank database were used for phylogenetic analysis. The reference viruses included 64 global PRRSV-1 strains, four Korean PRRSV-1 isolates, and two PRRSV-1 vaccine strains (Unistrain PRRS and Porcilis PRRS). These two vaccines have been commercially used since 2014 in Korea. Because Unistrain PRRS and Amervac PRRS contain common strain, VP-046 BIS, available nucleotide sequence of Amervac PRRS (accession number: GU067771) was used for Unistrain PRRS. For Porcilis PRRS, the nucleotide sequence of MLV-DV (accession number: KJ127878) was used. ORF2–7 sequences of the 27 KU viruses herein (KU-E1201–KU-E1208, KU-E1301–KU-E1303, KU-E1501–KU-E1505, KU-E1601–KU-E1604, KU-E1701–KU-E1703, KU-E1705, and KU-E1706–KU-E1709) were aligned with those of the reference viruses using MUSCLE (Edgar, 2004). ORF2–7-, and ORF5-, and ORF7-based phylogenetic trees were constructed by the maximum-likelihood method using the general time reversible model with gamma-distributed rate (four rate categories, Γ4) and invariant site (I), using MEGA6 (Tamura et al., 2013), and were evaluated by 1000 bootstrap replicates.

2.3. Bioinformatics

Two datasets were prepared as follows: before vaccine introduction (11 KU viruses; KU-E120x and KU-E130x) and after vaccine introduction (16 KU viruses; KU-E150x, KU-E160x, KU-E170x and CBNU0495). The genetic distances of amino acid sequences were calculated as pairwise distances (p-distance) using MEGA6. Statistical analysis of p-distance before and after vaccination was performed using the Mann-Whitney test. The action of selection pressure on the structural proteins of KU viruses was determined as the ratio of non-synonymous to synonymous substitution rates (dN/dS). The dN/dS ratio and site-by-site selection at single codon sites of each structural protein were estimated using single-likelihood ancestor counting, fixed-effects likelihood, and a mixed effects model of evolution available at DataMonkey (http://www.datamonkey.org) (Kosakovsky Pond and Frost, 2005a, b; Murrell et al., 2012). Codons that were confirmed by two or three different methods were identified as positively selected sites. Potential N-glycosylation sites in GP2a, GP3, GP4, and GP5 of PRRSV-1 were determined using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) (Gupta et al., 2004).

Table 1
List of primer sequences used in this study.

Use	Primer name[a]	Sequence	Product size
RT	PRRSV L15030R	5′-TCG CCC TAA TTG AAT AGG TGA-3′	–
PCR – fragment 1	PRRSV L11724F	5′-CTG GCA CAG AAT TGC AGG TG-3′	1844 bp
	PRRSV L13527R	5′-CCA AAA GCA AGA GTG CGG AA-3′	
PCR – fragment 2	PRRSV L13482F	5′-CAT YGC TTG TTT GTT CGC CAT-3′	1591 bp
	PRRSV L15030R	5′-TCG CCC TAA TTG AAT AGG TGA-3′	

[a] Primers were named based on nucleotide position in the Lelystad strain (M96262).

Table 2
Identities of nucleotides and amino acids between KU viruses (n = 27) and Lelystad and among Korean field strains.

Nucleotide			Amino acid		
	Lelystad	Korean field strains (n = 31)[a]		Lelystad	Korean field strains (n = 31)[a]
ORF2–7	88.9 ± 0.5 (88.0–90.1)	91.9 ± 1.7 (88.7–99.9)	–	–	–
ORF2a	90.4 ± 1.0 (88.1–92.0)	91.5 ± 2.7 (86.1–99.7)	GP2a	90.4 ± 1.1 (88.7–93.1)	91.5 ± 2.5 (85.5–99.5)
ORF2b	91.3 ± 1.2 (88.2–92.9)	94.8 ± 1.6 (89.2–100)	GP2b	94.4 ± 1.7 (88.5–95.7)	97.7 ± 2.0 (91.4–100)
ORF3	85.4 ± 1.0 (82.0–87.2)	89.5 ± 2.7 (81.9–100)	GP3	81.1 ± 1.4 (78.1–83.0)	86.3 ± 3.8 (76.6–100)
ORF4	86.4 ± 1.2 (82.0–88.4)	90.6 ± 2.7 (82.9–100)	GP4	84.8 ± 1.5 (81.4–87.4)	89.5 ± 2.9 (81.4–100)
ORF5	87.5 ± 1.1 (84.9–89.6)	91.9 ± 2.0 (87.7–100)	GP5	87.3 ± 1.3 (84.5–89.5)	91.1 ± 2.3 (84.0–100)
ORF5a	95.1 ± 1.4 (91.6–96.9)	95.2 ± 1.9 (87.8–100)	ORF5a	94.1 ± 2.2 (88.3–97.6)	94.4 ± 3.0 (83.7–100)
ORF6	90.2 ± 0.8 (88.6–91.5)	92.8 ± 1.5 (89.8–100)	M	94.0 ± 1.5 (90.1–95.9)	93.2 ± 1.9 (87.8–100)
ORF7	93.1 ± 1.5 (91.2–100)	95.1 ± 1.2 (91.2–100)	N	92.6 ± 1.7 (88.2–94.5)	96.7 ± 1.8 (89.8–100)

[a] Korean field strains included 27 KU viruses, KNU-07 (FJ349261), E38 (KT033457), D40 (KY434184) and CBNU0495 (KY434183).

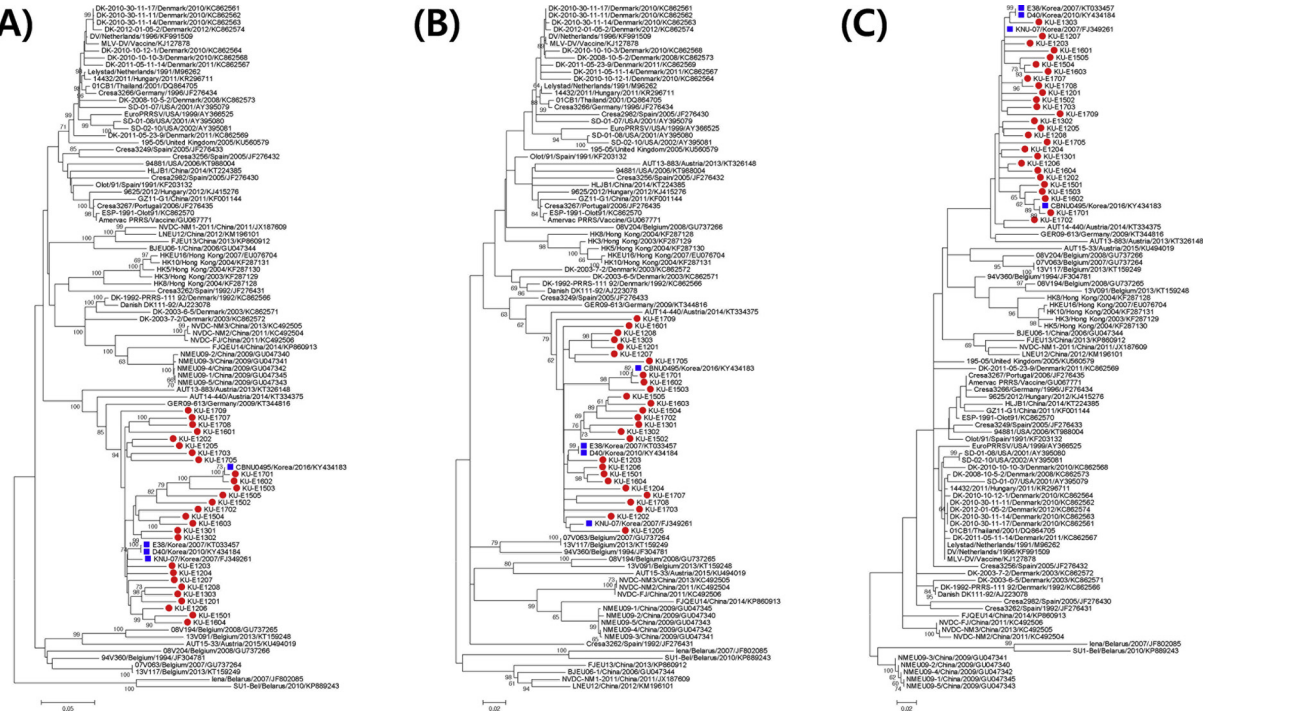

Fig. 1. Phylogenetic trees based on ORF2–7 (A), ORF5 (B), and ORF7 (C) of porcine reproductive and respiratory syndrome virus (PRRSV)-1. The trees were constructed using the maximum-likelihood method based on the general time reversible (GTR) model with G + I, and they were tested using 1000 bootstrapping values. Red circles and blue squares indicate KU viruses and Korean field strains, respectively.

3. Results

3.1. Genetic diversity and phylogenetic analysis of PRRSV-1

To explore the genetic characteristics of PRRSV-1 circulating in Korean pig herds before and after vaccine introduction, ORF2–7, covering whole structural proteins, of twenty-seven PRRSV-1 were sequenced. Nucleotide analysis indicated that the lengths of ORF2–7 of KU viruses were 3189 (16/27 viruses), 3186 (7/27), 3183 (1/27), 3177 (1/27), 3171 (1/27), and 3144 (1/27) nucleotides (nt), respectively, whereas those of Lelystad virus (LV, PRRSV-1 prototype) and KNU-07 (Korean strain isolated in 2007) were 3189 nt. Most deletions were located in an overlapping sequence encoding GP3 and GP4, in which nucleotide deletions have been commonly found in many other PRRSV-1 isolates (Oleksiewicz et al., 2000). Two viruses (KU-E1207 and KU-E1709) contained three nucleotide deletions in the 5′ terminus of ORF6.

ORF2–7 of KU viruses had a low level of nucleotide identity to that of LV (88.9 ± 0.5%, 88.0–90.1%). When comparing ORF2–7 of all Korean viruses (KNU-07, e38, and KU viruses), these strains were found to share 91.5 ± 1.7% (87.3–98.9%) nucleotide identity, suggesting that Korean viruses are genetically closer to each other than to the PRRSV-1 prototype. Among structural genes, ORF3 was the most variable region in Korean PRRSV-1, followed by ORF4, ORF5, and ORF2a. Unlike ORFs encoding glycoproteins, ORF7 was conserved at the nucleotide level. Analysis of amino acid sequences showed that nucleotide identity in GP3 was lowest among the structural proteins of Korean PRRSV-1. Nucleotide and amino acid identities are summarized in Table 2.

An ORF2–7-based phylogenetic tree indicated that KU viruses formed a distinct cluster with other Korean viruses, defined by a high bootstrap value (94%), despite the relatively high genetic variation (up to 10%) among Korean PRRSV-1 strains (Fig. 1A). They also were found to be closely related to German and two Austrian strains. However, KU viruses were phylogenetically distant from Porcilis PRRS and Unistrain PRRS that were commercially available vaccine strains in Korea since 2014 and shared 88.3 ± 0.5% (87.4–89.4%) and 88.0 ± 0.5% (87.1–89.0%) pairwise nucleotide identity to Porcilis PRRS and Unistrain PRRS, respectively. Furthermore, the ORF5-based phylogenetic tree was similar to the ORF2–7-based tree, but it had a lower bootstrapping value of 62% (Fig. 1B). In terms of the ORF7-based tree,

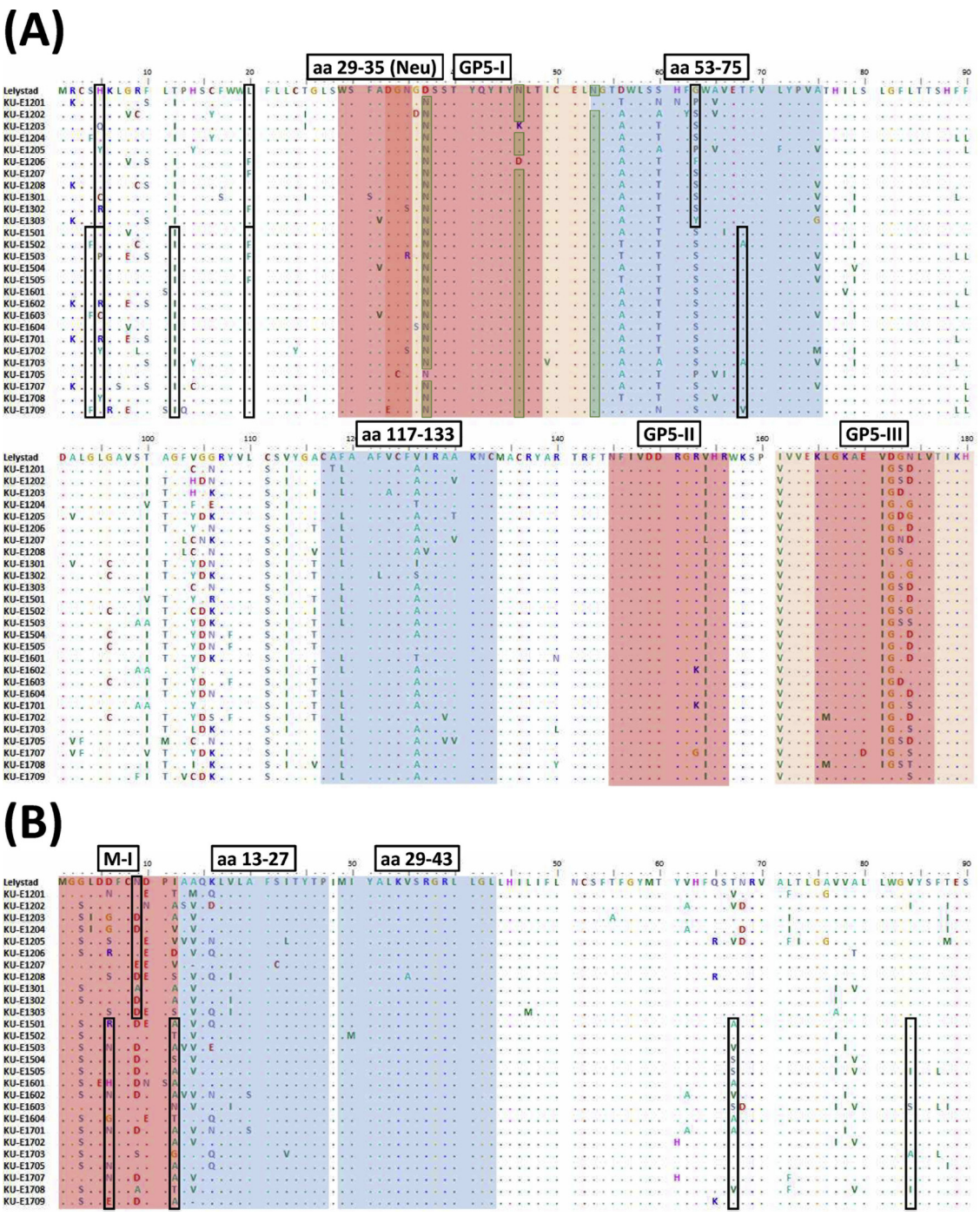

Fig. 2. Alignment of amino acid sequences of major structural proteins of KU viruses. (A) aa 1–180 of GP5 and (B) aa 1–90 of M. Red shade: previously identified B-cell epitope; blue shade: T-cell epitope; green shade: potential glycosylation site; black box: positively selected sites.

although Korean viruses clustered together, they exhibited a closer relationship with the AUT-14-440 strain than did the ORF2–7-based tree and their bootstrap value was very low (18%; Fig. 1C).

3.2. Analysis of major structural proteins (GP5, M, and N)

Previous studies identified two T-cell epitopes and five B-cell epitopes, including one neutralizing epitope at amino acid (aa) 29–35 in GP5 (Diaz et al., 2009; Mokhtar et al., 2014; Vanhee et al., 2011; Wissink et al., 2003). The neutralizing epitope was conserved among KU viruses (Fig. 2A). GP5-III was found to be variable, but other B-cell epitopes were conserved. Moreover, the significant increase in p-distance after vaccination was not identified in GP5 epitopes (Table 3). The cysteine residue at position 50 that participates in the formation of a heterodimer with the M protein was conserved in all KU virus (Verheije et al., 2002). A hypervariable region (aa 100–106) was identified within a predicted short ectodomain that is located between two transmembrane helix motifs (Dokland, 2010). One interesting observation was that a putative T-cell epitope at position 53–75 contained two positive selection sites at positions 63 and 68 (Table 4). In addition, N-linked glycosylation of GP5 was highly conserved at positions 37, 46, and 53, but some viruses were not found to harbor one of these glycosylation sites.

Cysteine residue at position 8 within a predicted N-terminal

Table 3
Comparison of pairwise distance (p-distance) of epitopes before and after vaccine introduction.

Protein	Epitope	Before	After	p-value
GP2	Total	0.066	0.095	< 0.000
	GP2-I	0.103	0.232	< 0.000
	GP2-II	0.076	0.192	< 0.000
	GP2-III	0.042	0.028	0.007
	GP2-IV	0	0.035	< 0.000
	GP2-V	0.015	0.062	< 0.000
GP3	Total	0.102	0.150	< 0.000
	GP3-I	0.110	0.170	< 0.000
	GP3-II	0	0.211	< 0.000
	GP3-III	0.170	0.219	0.003
	GP3-IV	0.015	0.068	< 0.000
	GP3-V	0	0.088	< 0.000
	GP3-VI	0.086	0.078	0.62
	GP3-VII	0	0	–
	GP3-VIII	0.342	0.371	0.058
GP4	Total	0.087	0.100	< 0.000
	aa 7-15	0.061	0.124	0.004
	GP4-I	0.564	0.642	< 0.000
	aa 173-203	0.017	0	< 0.000
GP5	Total	0.092	0.093	0.524
	aa 29-35	0.078	0.081	0.83
	GP5-I	0.029	0	< 0.000
	aa 53-75	0.124	0.105	0.19
	aa 117-133	0.119	0.087	0.004
	GP5-II	0.015	0.027	0.05
	GP5-III	0.136	0.143	0.695
	GP5-IV	0.081	0.057	0.016
M	Total	0.072	0.062	< 0.000
	M-I	0.317	0.235	< 0.000
	aa 13-27	0.136	0.119	0.063
	aa 29-43	0.012	0.009	0.243
	M-II	0.015	0.010	0.243
	M-III	0.015	0	< 0.000
	aa 153-173	0	0.040	< 0.000

Table 4
Selection pressure profiles before and after vaccine introduction.

Protein	Period	Mean dN/dS	Positively selected sites	
			N	Amino acid position (based on LV)
GP2	Before	0.298	3	**42**[a], 129, 184
	After	0.256	10	2[a], 10[a], 14, 21, 23, 28, 29, 36[a], **41**[a], **42**[a], 249
GP3	Before	0.390	4	6, 27, 93[a], 260[a]
	After	0.474	20	3, 6, 7, 9, 11, 12, 15, 27, 28, 30, 80[a], 96[a], 139, 166, 232[b], 243, **249**[a], **250**[a], **251**[a], 260[a]
GP4	Before	0.382	4	18, 49[a], 56[a], **65**[a]
	After	0.344	5	33, 51[a], **64**[a], **65**[a], **66**[a], **68**[a]
GP5	Before	0.426	3	5, 20, 63[c]
	After	0.255	5	4, 5, 12, 20, 68[c]
M	Before	0.328	1	9[a]
	After	0.222	4	6[a], 12[a], 67, 84
N	Before	0.182	0	N/D[d]
	After	0.186	0	N/D

[a] Sites for previously identified B-cell epitopes. Bold indicates sites in neutralizing epitopes.
[b] Potential glycosylation sites.
[c] Sites of previously identified T-cell epitopes.
[d] Not detected.

ectodomain plays a critical role in the formation of this heterodimer. This cysteine was found to be conserved among all KU viruses. In contrast, genetic variation was identified in the N-terminal ectodomain, which was also identified as a B-cell epitope (Fig. 2B). Whereas genetic distance of the epitope was decreased after vaccination, the epitope contained three positively selected sites at positions 9 (before vaccination), 6, and 12 (after vaccination). Three T-cell epitopes and two B-cell epitopes were also found to be conserved.

3.3. Analysis of minor structural proteins (GP2a, GP3, and GP4)

For GP2a, five B-cell epitopes, including two neutralizing epitopes, were previously identified (Vanhee et al., 2011). Of these, amino acid substitutions were found within the GP2-I epitope and GP2-II neutralizing epitope (Fig. 3A). Specifically, these epitopes became more variable and harbored more positively selected codons after vaccination. Moreover, the glycosylation pattern of GP2a was highly conserved among KU viruses, except KU-E1601, at position 173.

GP3 was found to be the most diverse structural protein of KU viruses. The biggest differences in average p-distances and dN/dS ratios between strains isolated before and after vaccination were identified in GP3. One previous study identified a large number of B-cell epitopes in GP3 (GP3-I to VIII) (Vanhee et al., 2011). The average p-distances of five consecutive epitopes, GP3-I to GP3-V, were considerably increased after vaccination. Whereas GP3-I and GP3-II were conserved before vaccination, they became variable among KU viruses after vaccination. GP3-II included a positively selected site at position 80 and GP3-III contained two positively selected sites at positions 92 and 95. The most variable epitope was GP3-VIII, which was identified as a neutralizing epitope (Fig. 3B) (Vanhee et al., 2011). For GP3-VIII, genetic variability between strains isolated before and after vaccination was not significant; however, we determined that residues at positions 249, 250, and 251 evolved under positive selection after vaccination. Compared to putative glycosylation sites in LV, we found that KU viruses had gained five new glycosylation sites (N29, N159, N230, N232, and N243). In KU viruses, the putative glycosylation pattern became more diverse after vaccination; specifically, the frequencies of N29 and N232 decreased, whereas N27, N230, and N243 appeared. N232 was also identified as a positively selected site.

In PRRSV-1, GP4 is considered a major target for neutralizing antibodies, which might be a driving force in the emergence of neutralizing antibody-resistant variants (Costers et al., 2010b). The neutralizing epitope was first recognized at position 40–79, and subsequent studies identified aa 57–68 as the core of this epitope (Meulenberg et al., 1997; Oleksiewicz et al., 2001; Vanhee et al., 2010). In KU viruses, the GP4 epitope exhibited the highest genetic diversity among all epitopes of structural proteins, and its genetic heterogeneity increased after vaccination. In addition, sites found to be under positive selection after vaccination (at positions 64, 65, 66 and 68) were located in the core epitope and some viruses acquired new glycosylation sites (N58 and N63) after vaccination (Fig. 3C).

4. Discussion

Despite the importance of vaccination in viral evolution, there is no information regarding the relationship between PRRSV evolution and vaccination due to the complexities and difficulties associated with this area of research. In the present study, whole structural genes were sequenced to assess the evolution of recently identified PRRSV-1 because minor structural proteins, in addition to major structural proteins, participate in viral pathogenesis and host-virus interactions. In addition, we also compared genetic characteristics before and after the introduction of PRRSV-1-based vaccines to avoid the following limitations. First, it is extremely challenging to determine the extent of vaccine-induced immunity in pigs infected with circulating field strains. This is because Korean farms have applied different vaccination strategies depending on the status of PRRS types in affected farms. In addition, the low level of biosecurity and close proximity in pig farming-dense areas may contribute to the frequent farm-to-farm transmission of PRRSV. Hence, current vaccination strategies cannot fully represent viral evolution under vaccine-induced immune pressure. The main findings of this study were as follows: 1) there has been an independent evolution of Korean PRRSV-1 strains and 2) there has been significant differential evolution within neutralizing epitopes of minor structural proteins after the introduction of PRRSV-1-specific vaccines.

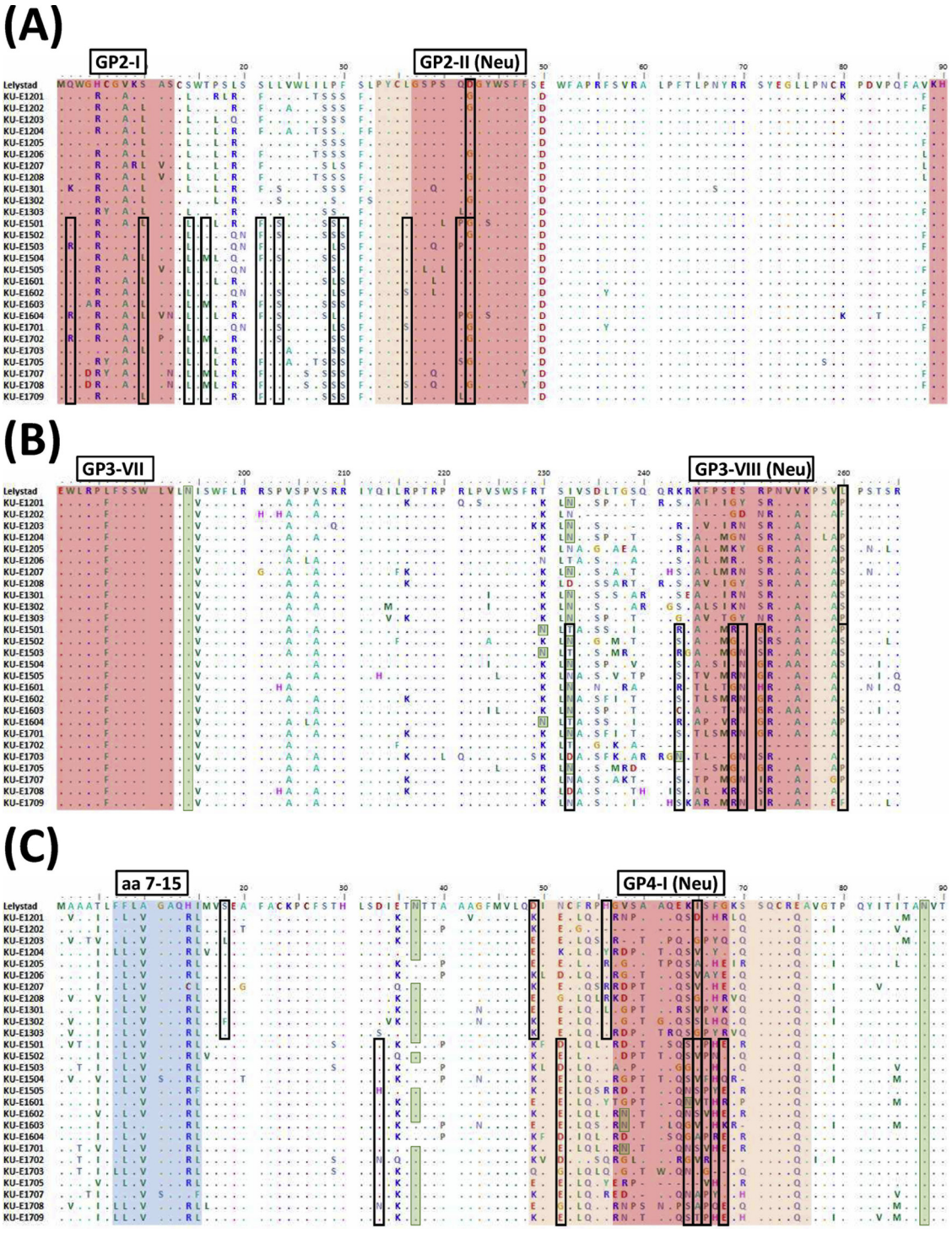

Fig. 3. Alignment of amino acid sequences of major structural proteins of KU strains. (A) aa 1–90 of GP2a, (B) aa 181–255 of GP3, and (C) aa 1–90 of GP4. Previously identified B-cell epitope; blue shade: T-cell epitope; green shade: potential glycosylation site; black box: positively selected sites.

PRRSV has been considered one of the most rapidly evolving viruses with a highly diverse genome (Hanada et al., 2005). The majority of its diversity was identified among PRRSV-1 strains in Europe, but the virus has been introduced and locally diversified in five non-European countries including Korea (Shi et al., 2010a). The first report of Korean PRRSV-1 that was identified in 2005 revealed 93% nucleotide identity to LV based on a partial ORF1b sequence. Subsequent studies identified that Korean PRRSV-1 strains are highly divergent from viruses from other countries and share a low level of identity to each other based on ORF5 (Kim et al., 2006, 2010; Nam et al., 2009). However, it was difficult to understand how PRRSV-1 had evolved in the field because previous studies provided only short nucleotide sequences or limited numbers of sequences from the complete genome. In addition, it was recently shown that two Austrian strains and one German strain are closely related to the KNU-07 strain based on the phylogenetic tree (Sinn et al., 2016). This raised the question of whether PRRSV-1 had evolved independently in Korea. In the present study, the approach using whole structural genes with increased sample size confirmed that Korean viruses shared a low level of genetic identity but formed their own clade. In addition, three strains (as state above) were identified as

located outside of the Korean clade in ORF2–7 phylogeny. Collectively, it is plausible that Korean PRRSV-1 had independently evolved after its introduction into Korea. Continuous surveillance with increased sample sizes is needed to provide the information about the continuous independent evolution as well as the possible emergence of novel PRRSV-1. Whereas similar results were obtained from the ORF5-based tree, one Austrian strain (AUT14-440) formed a group together with Korean viruses in the ORF7-based tree, suggesting that the phylogenetic analysis of a single gene (ORF7) could not fully represent the molecular epidemiology of Korean PRRSV-1.

GP5 is considered a prime target for antibody-mediated neutralization of PRRSV-2 (Ostrowski et al., 2002; Wissink et al., 2003). Our analysis demonstrated considerable genetic diversity among major structural proteins of KU viruses. However, the GP5 neutralizing epitope was conserved, and no significant differences in terms of genetic diversity before and after vaccination were identified. Interestingly, the hypervariable region of GP5 and N-terminus of the M protein, which were identified as putative ectodomains, were variable among KU viruses. In PRRSV-2, residues within these corresponding ectodomains play key roles in determining susceptibility to viral neutralization by polyclonal swine antibodies (Fan et al., 2015; Trible et al., 2015).

Current studies on the molecular epidemiology of PRRSV have focused on the genetic characteristics of ORF5 and/or ORF7. Accordingly, this study broadens the genetic information regarding circulating PRRSV-1 strains in the field. Minor structural proteins of KU viruses shared a low-level of identity each other; in addition, multiple antigenic regions within minor structural proteins were found to be genetically diverse. Especially, our findings showed a statistically significant increase in genetic distance, an increased number of sites subjected to diversifying evolution, and the emergence of novel glycosylation sites in the neutralizing epitopes of minor structural proteins after vaccination. Currently, it is well-known that antibodies against the GP4 epitope are highly immunogenic and have potent neutralizing activity (Vanhee et al., 2011). Under immunoselection by neutralizing monoclonal antibodies against the GP4 epitope *in vitro*, this virus was previously found to acquire the ability to escape neutralizing antibodies *via* amino acid substitutions within the GP4 epitope (Costers et al., 2010a). Further, during infection *in vivo*, vaccination was found to result in the emergence of antibody-escaping mutants, in which strong positive selection contributed to amino acid substitutions in the GP4 neutralizing epitope (Costers et al., 2010b). In this regard, it is possible that vaccine-induced immunity directs enhanced diversifying evolution of the GP4 epitope with increased genetic heterogeneity in the field. In addition, it has been established that PRRSV escapes the host immune response through a glycan shielding mechanism, in which the loss of glycosylation enhances the sensitivity of PRRSV to neutralization (Ansari et al., 2006; Vu et al., 2011). For KU viruses, the novel emergence of two potential glycosylation sites occurred in the core of the neutralizing epitope after the introduction of the vaccine. In a previous study, vaccination induced the emergence of an antibody escape mutant containing N65 in the GP4 neutralizing epitope (Costers et al., 2010b). The effect of new glycosylation sites in the neutralizing epitope on PRRSV survival has not been investigated yet; however, these facts suggest the possibility that PRRSV could evade antibody-mediated neutralization through the glycan shielding of GP4, with the introduction of vaccination. In contrast to the GP4 epitope, knowledge on the neutralizing characteristics of GP2a and GP3 remains limited. GP2a and GP3 neutralizing epitopes were less immunogenic and antibodies against these epitopes were found to have weaker neutralizing activities compared to those targeting the GP4 epitope (Vanhee et al., 2011). Our results showed increased genetic distance and enhanced diversifying evolution of GP2a and GP3 neutralizing epitopes. These significant changes highlight the importance of multiple antigenic regions in the evolution of PRRSV-1 in the vaccination era. Although the clinical implications of those changes were not elucidated, the effect of changes within neutralizing epitopes of minor structural proteins on clinical manifestation in the field should be further evaluated.

5. Conclusion

In conclusion, this study presents, for the first time, an investigation of the viral structural proteins of recently identified Korean PRRSV-1 strains, in addition to a comparison of diversity between 11 sequences from before introduction and 17 from after vaccine introduction. Whole structural genes of Korean PRRSV-1 have independently evolved in the commercial pig population. After the introduction of PRRSV-1 vaccines, increased genetic heterogeneity, enhanced diversifying evolution, and the emergence of novel glycosylation sites were found within neutralizing epitopes of minor structural proteins. This work provides novel insight into the evolution of PRRSV and furthermore could contribute to the knowledge on the antigenicity and immune evasion strategies of this virus.

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