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ORIGINAL ARTICLE

Independent evolution of porcine reproductive and respiratory syndrome virus 2 with genetic heterogeneity in antigenic regions of structural proteins in Korea

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pathogen that affects the global swine industry. The continuous evolution of this virus has made control and prevention difficult, which emphasizes the importance of monitoring currently circulating PRRSV strains. In this study, we investigated the genetic characteristics of whole structural genes of 35 PRRSV-2 isolates that circulated between 2012 and 2017 in Korea. Genetic and phylogenetic analysis demonstrated that a recently identified PRRSV-2 shared a relatively low level of nucleotide sequence identity that ranged from 86.2% to 92.8%; however, they were clustered into four distinct Korean field clades, except KU-N1702, in ORF2–7-based phylogeny. KU-N1702 was closely related to the NADC30-like strains that were identified in the USA and China. Amino acid sequence analysis showed that the GP5 neutralizing epitope was conserved among the KU viruses. In contrast, the viruses had genetic mutations in key residues for viral neutralization within GP5 and M. For minor structural proteins, neutralizing epitopes, aa 41–55 of GP2, 61–75 of GP3, and 51–65 of GP4, were variable among the KU viruses. Bioinformatics demonstrated diversifying evolution within the GP2 and GP4 neutralizing epitopes and the emergence of a novel glycosylation site within the GP3 and GP4 neutralizing epitopes. Taken together, these data provide evidence that Korean PRRSV-2 evolved independently in Korea, with genetic heterogeneity in antigenic regions of structural proteins.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a very important pathogen that causes huge economic losses in the swine industry worldwide. PRRS were first documented in the United States in the late 1980s, and the virus was isolated in Europe in 1990 [8, 44]. After identifying this virus as the causative agent of severe reproductive losses and respiratory distress in pigs, a large number of clinically similar outbreaks occurred worldwide [49]. PRRSV is an enveloped, single-stranded, positive-sense RNA virus belonging to the order *Nidovirales* and family *Arteriviridae*. The genome of PRRSV contains 10 open reading frames (ORFs) and is approximately 15 kb in length. ORF1a and ORF1b encode two large polyproteins that are proteolytically cleaved into 14 active nonstructural proteins [19]. Eight ORFs (ORF2a, ORF2b, ORF3–7, and ORF5a) encode eight structural proteins, including glycoprotein (GP) 2, small envelope (E), GP3, GP4, GP5, membrane (M), nucleocapsid (N), and ORF5a proteins, respectively [21, 46]. Two major envelope proteins, GP5 and M, form a disulfide-linked heterodimer that is essential for virion formation [25]. GP2, GP3, and GP4 form a trimeric envelope protein complex that is involved in viral entry through its interaction with CD163 of the host cell [10, 33].

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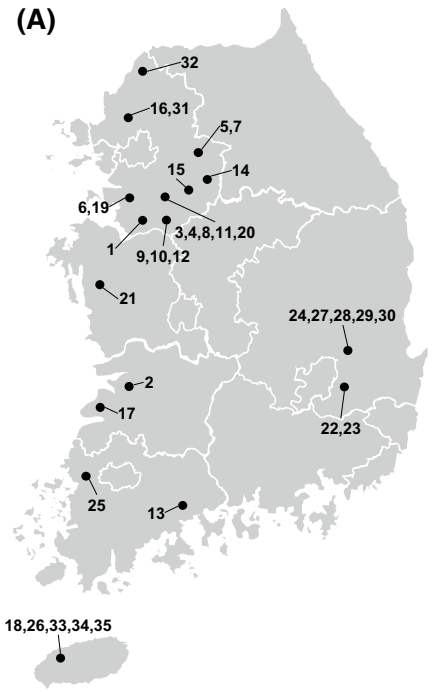
PRRSV is divided into two types: PRRSV-1 (European and type 1) and PRRSV-2 (North American and type 2), which belong to separate species [23]. The two PRRSV types vary by approximately 40% in their nucleotide sequences. There is considerable genetic diversity among PRRSV-1 and PRRSV-2 isolates, with genetic variation up to 30% and 20%, respectively, in their ORF5 sequences [49]. This high level of genetic variation arises from three mechanisms: 1) error-prone RNA polymerase activity, 2) natural selection, and 3) viral recombination after coinfection with two different PRRSV strains in one cell [3]. The emergence of novel PRRSV strains occurs continually, especially for PRRSV-2. Examples include an outbreak of 'acute PRRS' in 1996, an outbreak of virulent MN184 in 2001, and an outbreak of a highly pathogenic PRRSV in 2006 [4, 18, 35]. Large-scale phylogenetic analysis of ORF5 sequences revealed that PRRSV-2 can be divided into nine well-established lineages [31].

To date, most studies on the molecular epidemiology of PRRSV have focused on ORF5 due to its high genetic variability and the discovery of a neutralizing epitope. However, this analysis might not represent the molecular epidemiology and genetic evolution of PRRSV-2 because ORF5 comprises only approximately 5% of the full-length genome of PRRSV. In addition, previous reports showed that antibodies against the linear epitope of the GP5 ectodomain were unable to suppress infection by PRRSV-1 and PRRSV-2 in porcine alveolar macrophages (PAM) [24, 38]. However, recent studies have provided important information regarding the role of the minor envelope proteins in PRRSV biology. Minor structural proteins interact with CD163 molecules and determine host cell binding and viral tropism *in vitro* [10, 33]. This information provides novel insights into the function of these minor glycoproteins and suggests the possibility that they could serve as targets for neutralizing antibodies. In PRRSV-1, several regions of minor structural proteins are known to be targeted by neutralizing antibodies [9, 37]. Therefore, the goal of this study was to determine the genetic and evolutionary characteristics of viral structural proteins of PRRSV-2 currently circulating in Korea.

Materials and methods

RT-PCR and sequence analysis

Thirty-five clinical samples (serum and lung) collected from different conventional Korean pig farms nationwide were used in this study (Fig. 1). All samples were identified as positive for PRRSV-2 through routine diagnostics



(B)

No.	Name	Year	Sample	Location of farm (city, province)	ORF2–7 clade	ORF5 lineage
1	KU-N1201	2012	Serum	Pyeongtaek, Gyeonggi	II	N/D (II)[a]
2	KU-N1203	2012	Serum	Gimje, Jeollabuk	I	L1
3	KU-N1204	2012	Serum	Yongin, Gyeonggi	I	L1
4	KU-N1205	2012	Serum	Yongin, Gyeonggi	I	L1
5	KU-N1206	2012	Serum	Yangpyeong, Gyeonggi	II	N/D (II)
6	KU-N1301	2013	Serum	Hwaseong, Gyeonggi	I	L1
7	KU-N1302	2013	Serum	Yangpyeong, Gyeonggi	I	L1
8	KU-N1303	2013	Serum	Yongin, Gyeonggi	II	N/D (II)
9	KU-N1304	2013	Serum	Anseong, Gyeonggi	II	N/D (II)
10	KU-N1305	2013	Serum	Anseong, Gyeonggi	II	N/D (II)
11	KU-N1306	2013	Serum	Yongin, Gyeonggi	I	L1
12	KU-N1307	2013	Serum	Anseong, Gyeonggi	I	L1
13	KU-N1308	2013	Serum	Suncheon, Jeollanam	II	N/D (II)
14	KU-N1401	2014	Serum	Yeoju, Gyeonggi	I	L1
15	KU-N1501	2015	Serum	Icheon, Gyeonggi	II	N/D (II)
16	KU-N1601	2016	Serum	Yangju, Gyeonggi	I	N/D
17	KU-N1602	2016	Serum	Buan, Jeollabuk	II	N/D (II)
18	KU-N1603	2016	Serum	Jeju	IV	L5.1
19	KU-N1604	2016	Lung	Hwaseong, Gyeonggi	I	L1
20	KU-N1605	2016	Lung	Yongin, Gyeonggi	I	N/D[b]
21	KU-N1701	2017	Serum	Hongseong, Gyeonggi	III	N/D (III)[c]
22	KU-N1702	2017	Lung	Gyeongsan, Gyeongsangbuk	NADC30-like	L1
23	KU-N1703	2017	Lung	Gyeongsan, Gyeongsangbuk	II	N/D (II)
24	KU-N1704	2017	Serum	Yeongcheon, Gyeongsangbuk	I	L1
25	KU-N1705	2017	Serum	Hampyeong, Jeollanam	I	L1
26	KU-N1706	2017	Serum	Jeju	IV	L5.1
27	KU-N1707	2017	Serum	Yeongcheon, Gyeongsangbuk	I	L1
28	KU-N1708	2017	Serum	Yeongcheon, Gyeongsangbuk	I	L1
29	KU-N1709	2017	Serum	Yeongcheon, Gyeongsangbuk	I	L1
30	KU-N1710	2017	Serum	Yeongcheon, Gyeongsangbuk	I	L1
31	KU-N1711	2017	Lung	Yangju, Gyeonggi	III	N/D (III)
32	KU-N1712	2017	Serum	Yeoncheon, Gyeonggi	III	N/D (III)
33	KU-N1713	2017	Serum	Jeju	IV	L5.1
34	KU-N1714	2017	Serum	Jeju	IV	L5.1
35	KU-N1715	2017	Serum	Jeju	IV	L5.1

Fig. 1 (A) Locations of farms. (B) Sample and KU virus information. PRRSV-2 in this study originated from 35 different farms nationwide between 2012 and 2017. Dots indicate geographical locations of cities where samples were collected. [a] The lineage of clade II was not determined based on ORF5 phylogeny. [b] The lineage of two viruses belonging to clade I based on ORF2–7 phylogeny was not determined based on ORF5 phylogeny. [c] The lineage of clade III was not determined based on ORF5 phylogeny

performed between 2012 and 2017, and samples that showed vaccine-like characteristics (over 98% nucleotide sequence identity to ORF2–7 of VR-2332) were excluded. Total RNA was extracted from the samples using QIAzol Lysis Reagent (QIAGEN, MD, USA) according to the manufacturer's instructions. cDNA was synthesized using a specific primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). To determine the nucleotide sequence of ORF2 to ORF7, covering all structural proteins of PRRSV-2, two sets of PRRSV-2-specific primers were used. PCR amplification was performed using Takara Ex Taq (TaKaRa Bio, Shiga, Japan) under the following conditions: 35 cycles of 10 s at 98 °C, 30 s at 53 °C, 2 min at 72 °C. The primers used in this study are listed in Table 1. After the identification of target bands on 1.2% agarose gels, amplified products were purified using a commercial gel extraction kit (DokDo-Prep™ Gel Extraction Kit (spin-type), ELPIS BIOTECH Inc., Daejeon, Korea). Purified DNA was submitted to a commercial sequencing facility for direct sequencing in both directions (Macrogen Inc., Seoul, Korea). ORF2–7 sequences of 35 PRRSV-2 (named Konkuk University virus and KU virus) in this study were deposited in the GenBank database under accession numbers KY996346–KY996364 and MG999913–MG999928.

Phylogenetic analysis

To investigate the molecular epidemiology of PRRSV-2, sequences of ORF2–7, which covers whole structural proteins from 35 KU viruses (KU-N1201, KU-N1203 to KU-N1206, KU-N1301 to KU-N1308, KU-N1401, KU-N1501, KU-N1601 to KU-N1605, and KU-N1701 to KU-1715), were aligned with those of 155 global PRRSV-2 isolates, including nine Korean PRRSV-2 isolates obtained from GenBank, using MUSCLE [14]. A phylogenetic tree based on ORF2–7 of PRRSV-2 was constructed by the maximum-likelihood method with the general time-reversible model with a gamma-distributed rate (four rate categories) and invariant sites using MEGA 6 [32]. The tree was statistically estimated by 1,000 replicates of bootstrap analysis. Additionally, to determine the genetic lineage of Korean PRRSV-2 isolate, an ORF5-based phylogenetic tree was created with 130 PRRSV-2 using the same method.

Bioinformatics

A significant recombination event was detected using two different programs: 1) Recombination Detection Program 4 and 2) Simplot software v 3.5.1. Gene-specific selective pressure for the structural proteins of Korean PRRSV-2 was evaluated as the ratio of non-synonymous and synonymous (dN/dS) substitution rates. The data set included each ORF2–ORF7 of 35 KU viruses. The dN/dS ratio and the selective pressure at individual codons was estimated for GP2, E, GP3, GP4, GP5, ORF5a, M, and N using single-likelihood ancestor counting, fixed-effects likelihood, and a mixed-effects model of evolution, available at the DataMonkey (http://www.datamonkey.org) [30]. Codons confirmed using two or more different methods were identified as sites that were subjected to positive selection. Putative N-glycosylation profiles of four glycoproteins of PRRSV-2 were predicted using NetNGlyc 1.0 server [17].

Table 1 List of primer sequences used in this study

Use	Primer name[a]	Sequence	Product size
RT	PRRSV V15343R	5′-TCG CCC TAA TTG AAT AGG TGA-3′	-
PCR – fragment 1	PRRSV V12056F	5′-GCC CTG TCA TTG AAC CAA C-3′	1824 bp
	PRRSV V13840R	5′-GAT AGA ACG GCA CGA TAC AC-3′	
PCR – fragment 2	PRRSV V13727F	5′-TCA TGA CAC CTG AGA CCA TG-3′	1658 bp
	PRRSV V15343R	5′-TCG CCC TAA TTG AAT AGG TGA-3′	

[a]Primers were named based on nucleotide position located in the VR-2332 (U87392)

Results

Genetic diversity of Korean PRRSV-2

To investigate the genetic diversity of PRRSV-2 circulating in Korean pig herds between 2012 and 2017, whole structural genes of 35 KU viruses were sequenced and analyzed. The ORF2–7 region of most KU viruses comprised 3188 nucleotides (nt) and was identical to that of the prototype VR-2332. KU-N1304 and KU-N1710 contained a 3-nt deletion and an insertion in the N protein, respectively. Amino acid sequence analysis showed that KU-N1304, KU-N1602, and KU-N1703 had an 11-, 1-, and 1-aa deletion, respectively, in the C-terminus of GP2 when compared to VR-2332. The truncated GP2 proteins of the two viruses resulted from the introduction of a stop codon, not from the nucleotide deletion.

Nucleotide sequence analysis showed that the ORF2–7 region of KU viruses shared 89.9 ± 1.5% (86.2–92.8%) identity to that of VR-2332. In addition, the pairwise nucleotide sequence identity between Korean field strains was 84.0–98.6% (88.1 ± 2.1%). The pairwise nucleotide and amino acid sequence identity was determined for each structural gene. All genes tested were genetically distinct from those of VR-2332, with approximately 90% identity. ORF5 was the most variable region among the structural genes, and ORF4 and ORF3 also exhibited a high level of genetic diversity. At the amino acid level, the M protein was the most conserved, whereas the ORF5a protein had the lowest identity. Interestingly, GP3 was more diverse than GP5 when comparing Korean field strains. Collectively, Korean PRRSV-2 exhibited high genetic diversity in the structural genes. The nucleotide and amino acid sequence identity values between KU viruses and VR-2332, and between Korean field strains are summarized in Table 2.

Phylogenetic analysis of Korean PRRSV-2

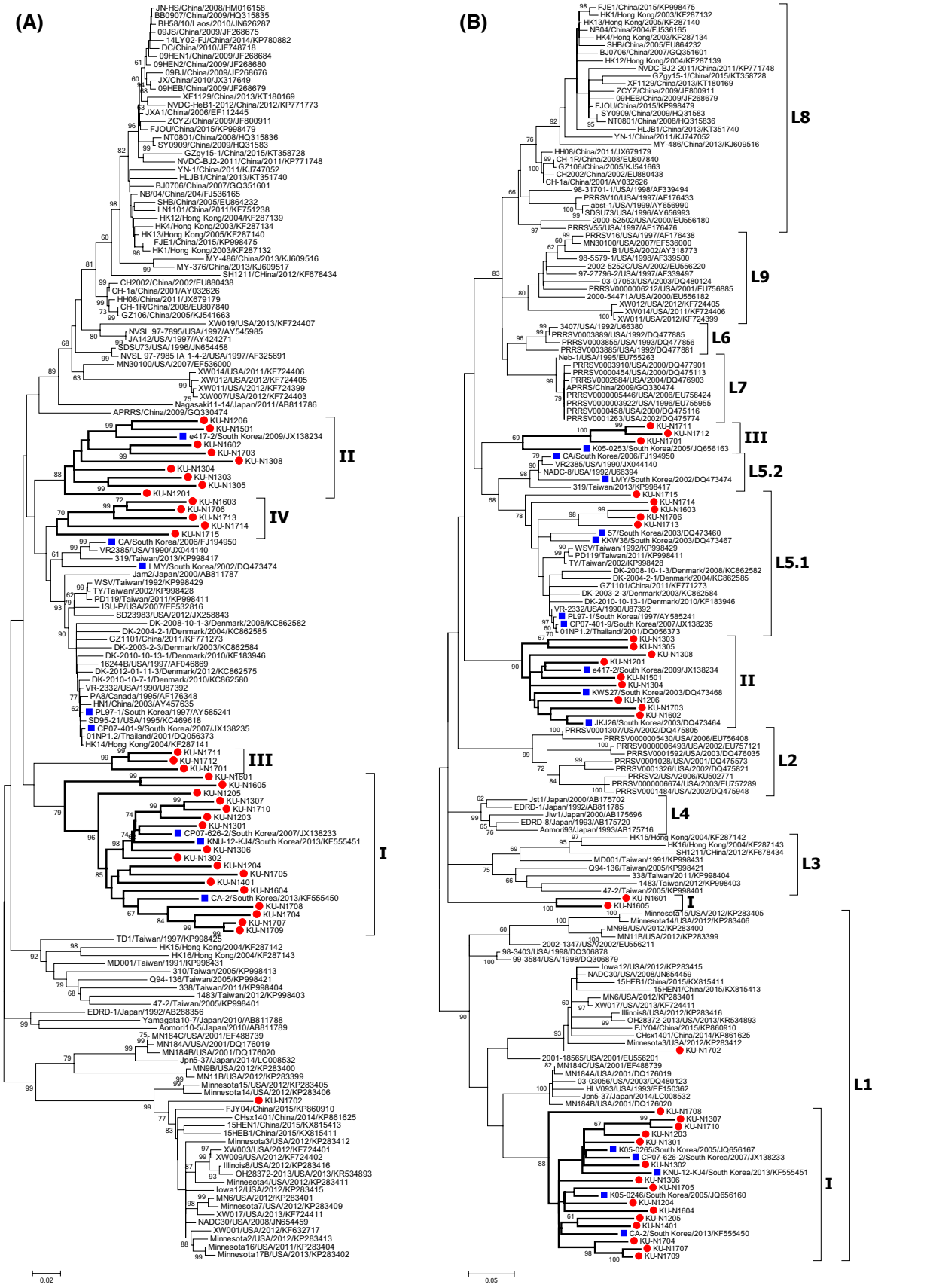
No significant recombination events were detected among the Korean PRRSV-2 isolates in this study (data not shown). A phylogenetic tree based on ORF2–7 of PRRSV-2 indicated that most Korean viruses formed distinct groups (Korean field clades I to IV; Fig. 2A). All clades were statistically supported in their phylogeny except KU-N1715 within Korean field clade IV. In contrast to the viruses that belonged to the Korean field clades, two Korean viruses, LMY and CA, which were identified in the early to mid-2000s, were

Fig. 2 Phylogenetic tree based on ORF2–7 (A) and ORF5 (B) of PRRSV-2. The trees were constructed using the maximum-likelihood method based on the general time-reversible model with a gamma-distributed rate (four rate categories) and invariant sites and tested using 1000 bootstrap replicates. Bootstrap values greater than 60 are shown. Lineages (L) were determined based on the lineage classification system of Shi et al. [31] Red circles and blue squares indicate KU viruses and Korean field strains, respectively

Table 2 Nucleotide and amino acid sequence identity between KU viruses (n = 35) and VR-2332 and between Korean field strains

	Nucleotide			Amino acid	
	VR-2332	Korean field strains (n = 41)[a]		VR-2332	Korean field strains (n = 41)[a]
ORF2–7	89.9 ± 1.5 (86.2–92.8)	88.1 ± 2.1 (84.0–98.6)		-	-
ORF2a	91.1 ± 1.9 (87.1–97.6)	88.4 ± 2.2 (82.3–99.2)	GP2a	90.4 ± 2.2 (85.1–93.7)	87.4 ± 2.7 (80.8–99.6)
ORF2b	93.1 ± 2.2 (89.6–97.7)	91.1 ± 2.4 (85.5–99.0)	GP2b	93.4 ± 2.4 (89.0–98.6)	92.3 ± 2.9 (82.1–100)
ORF3	89.1 ± 2.4 (83.0–94.9)	86.9 ± 2.7 (80.1–98.9)	GP3	87.1 ± 2.5 (80.7–92.1)	85.8 ± 3.2 (76.7–98.4)
ORF4	88.1 ± 1.5 (85.6–91.0)	88.9 ± 3.2 (81.5–99.8)	GP4	88.4 ± 2.9 (83.1–93.8)	89.6 ± 2.9 (83.7–100)
ORF5	86.8 ± 2.7 (83.7–93.3)	86.2 ± 3.0 (80.9–97.8)	GP5	86.0 ± 2.3 (82.5–92.0)	86.6 ± 3.5 (77.5–98.0)
ORF5a	81.9 ± 5.0 (75.0–91.9)	85.1 ± 6.2 (71.7–98.7)	ORF5a	81.7 ± 4.8 (76.4–92.1)	83.7 ± 7.0 (68.6–100)
ORF6	92.2 ± 2.8 (88.3–97.9)	91.4 ± 2.4 (85.5–98.2)	M	95.4 ± 1.8 (92.5–99.4)	94.9 ± 1.9 (90.8–99.4)
ORF7	92.7 ± 2.6 (88.1–99.1)	89.5 ± 2.5 (83.4–98.3)	N	94.2 ± 2.9 (87.8–100)	91.5 ± 3.3 (82.1–100)

[a]The Korean field strains included 35 KU viruses, LMY (DQ473474), CA (FJ194950), CP07-626-2 (JX138235), e417-2 (JX138234), CA-2 (KF555450), and KNU-12-KJ4 (KF555451)


(A)
JN-HS/China/2008/HM016158
BB0907/China/2009/HQ315835
BH58/10/Laos/2010/JN626287
09JS/China/2009/JF268675
14LY02-FJ/China/2014/KP780882
DC/China/2010/JF748718
09HEN1/China/2009/JF268684
09HEN2/China/2009/JF268680
09BJ/China/2009/JF268676
JX/China/2010/JX317649
09HEB/China/2009/JF268679
XF1129/China/2013/KT180169
NVDC-HeB1-2012/China/2012/KP771773
JXA1/China/2006/EF112445
ZCYZ/China/2009/JF800911
FJOU/China/2015/KP998479
NT0801/China/2008/HQ315836
SY0909/China/2009/HQ31583
GZgy15-1/China/2015/KT358728
NVDC-BJ2-2011/China/2011/KP771748
YN-1/China/2011/KJ747052
HLJB1/China/2013/KT351740
BJ0706/China/2007/GQ351601
NB/04/China/204/FJ536165
SHB/China/2005/EU864232
LN1101/China/2011/KF751238
HK12/Hong Kong/2004/KF287139
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FJE1/China/2015/KP998475
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CH2002/China/2002/EU880438
CH-1a/China/2001/AY032626
HH08/China/2011/JX679179
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XW014/USA/2011/KF724406
XW011/USA/2012/KF724399
L9
3407/USA/1992/U66380
PRRSV0003889/USA/1992/DQ477885
PRRSV0003855/USA/1993/DQ477856
PRRSV0003885/USA/1992/DQ477881
L6
Neb-1/USA/1995/EU755263
PRRSV0003910/USA/2000/DQ477901
PRRSV0000454/USA/2000/DQ475113
PRRSV0002684/USA/2004/DQ476903
APRRS/China/2009/GQ330474
PRRSV0000005446/USA/2006/EU756424
PRRSV0000003922/USA/1996/EU755955
PRRSV0000458/USA/2000/DQ475116
PRRSV0001263/USA/2002/DQ475774
L7
KU-N1711
KU-N1712
KU-N1701
III
K05-0253/South Korea/2005/JQ656163
CA/South Korea/2006/FJ194950
VR2385/USA/1990/JX044140
NADC-8/USA/1992/U66394
LMY/South Korea/2002/DQ473474
L5.2
319/Taiwan/2013/KP998417
KU-N1715
KU-N1714
KU-N1603
KU-N1706
KU-N1713
57/South Korea/2003/DQ473460
KKW36/South Korea/2003/DQ473467
WSV/Taiwan/1992/KP998429
PD119/Taiwan/2011/KP998411
TY/Taiwan/2002/KP998428
DK-2008-10-1-3/Denmark/2008/KC862582
DK-2004-2-1/Denmark/2004/KC862585
GZ1101/China/2011/KF771273
DK-2003-2-3/Denmark/2003/KC862584
DK-2010-10-13-1/Denmark/2010/KF183946
VR-2332/USA/1990/U87392
PL97-1/South Korea/1997/AY585241
CP07-401-9/South Korea/2007/JX138235
01NP1.2/Thailand/2001/DQ056373
L5.1
KU-N1303
KU-N1305
KU-N1308
KU-N1201
e417-2/South Korea/2009/JX138234
KU-N1501
KU-N1304
KWS27/South Korea/2003/DQ473468
KU-N1206
KU-N1703
KU-N1602
JKJ26/South Korea/2003/DQ473464
II
PRRSV0001307/USA/2002/DQ475805
PRRSV0000005430/USA/2006/EU756408
PRRSV0000006493/USA/2002/EU757121
PRRSV0001592/USA/2003/DQ476035
PRRSV0001028/USA/2001/DQ475573
PRRSV0001326/USA/2002/DQ475821
PRRSV2/USA/2006/KU502771
PRRSV0000006674/USA/2003/EU757289
PRRSV0001484/USA/2002/DQ475948
L2
Jst1/Japan/2000/AB175702
EDRD-1/Japan/1992/AB811785
Jiw1/Japan/2000/AB175696
EDRD-8/Japan/1993/AB175720
Aomori93/Japan/1993/AB175716
L4
HK15/Hong Kong/2004/KF287142
HK16/Hong Kong/2004/KF287143
SH1211/China/2012/KF678434
MD001/Taiwan/1991/KP998431
Q94-136/Taiwan/2005/KP998421
338/Taiwan/2011/KP998404
1483/Taiwan/2012/KP998403
47-2/Taiwan/2005/KP998401
L3
KU-N1601
KU-N1605
I
Minnesota15/USA/2012/KP283405
Minnesota14/USA/2012/KP283406
MN9B/USA/2012/KP283400
MN11B/USA/2012/KP283399
2002-1347/USA/2002/EU556211
98-3403/USA/1998/DQ306878
99-3584/USA/1998/DQ306879
Iowa12/USA/2012/KP283415
NADC30/USA/2008/JN654459
15HEB1/China/2015/KX815411
15HEN1/China/2015/KX815413
MN6/USA/2012/KP283401
XW017/USA/2013/KF724411
Illinois8/USA/2012/KP283416
OH28372-2013/USA/2013/KR534893
FJY04/China/2015/KP860910
CHsx1401/China/2014/KP861625
Minnesota3/USA/2012/KP283412
KU-N1702
2001-18565/USA/2001/EU556201
MN184C/USA/2001/EF488739
MN184A/USA/2001/DQ176019
03-03056/USA/2003/DQ480123
HLV093/USA/1993/EF150362
Jpn5-37/Japan/2014/LC008532
MN184B/USA/2001/DQ176020
L1
KU-N1708
KU-N1307
KU-N1710
KU-N1203
KU-N1301
K05-0265/South Korea/2005/JQ656167
CP07-626-2/South Korea/2007/JX138233
KU-N1302
KNU-12-KJ4/South Korea/2013/KF555451
KU-N1306
KU-N1705
K05-0246/South Korea/2005/JQ656160
KU-N1204
KU-N1604
KU-N1205
KU-N1401
CA-2/South Korea/2013/KF555450
KU-N1704
KU-N1707
KU-N1709
I
0.05

grouped with VR-2385. Two clades included most of the Korean field strains that were identified in this study. The Korean field clade I included 17 KU viruses as well as two Korean nsp2 deletion isolates, which were closely related to isolates commonly found in the USA [7]. Nine KU viruses were clustered with e417-2 in Korean field clade II. While viruses from mainland Korea belonged to clades I, II, and III, clade IV consisted of the viruses from Jeju Island, where the trade of live pigs with mainland Korea is prohibited. Notably, KU-N1702 did not belong to any Korean clade and shared a close relationship with US and Chinese viruses, of which NADC30 and 15HEB1 had 94.4% and 93.0% nucleotide sequence identity, respectively, to KU-N1702 (Table 3).

Korean field clades I, II, and III were well supported in ORF5-based phylogeny, but the viruses in clade IV were scattered throughout lineage 5.1 (Fig. 2B). According to the lineage classification of PRRSV-2 [31], Korean field clade I was classified into lineage 1. Interestingly, while KU-N1601 and KU-N1605 were associated with clade I based on the ORF2–7-based tree, the ORF5-based tree indicated that they were differently positioned. Furthermore, Korean field clades II and III had no close relationship to any existing lineages of PRRSV-2. Based on ORF5 sequences, viruses belonging to Korean field clades have been identified since 2003 or 2005 [5, 6].

Major structural proteins (GP5, M, and N) of Korean PRRSV-2

For GP5, many studies had previously identified the antigenic sites for antibody production and the stimulation of IFN-γ-secreting cells [12, 28, 39, 48]. While the decoy epitope at position 27–30 was highly variable among KU viruses, the neutralizing epitope at position 37–44 was conserved (Fig. 3A). Amino acid sequence diversity occurred in different regions as well as in three B-cell epitopes, namely, aa 1–15, 168–178, and 187–200, and one T-cell epitope, namely aa 117–131. Of the previously identified epitopes, it was determined that the decoy epitope and two B-cell epitopes (aa 1–15 and 186–200) had evolved under positive selection pressure. Regarding key residues that determine susceptibility to viral neutralization, the residue at position 102 was variable, and five KU viruses had a cysteine that was identical in position to that found in a neutralizing antibody-escape mutant [15]. The residue at position 104 was highly conserved; most KU viruses had glycine, but three viruses possessed glutamic acid, which had not been identified previously. Different potential glycosylation patterns were observed regarding the total number and positions of GP5 glycosylation sites for KU viruses. Twelve different glycosylation patterns were identified and major variations in putative glycosylation sites were mainly located between positions 30 and 35, in which four sites were also identified as being subjected to positive selection.

As the most conserved structural protein, 132 out of 174 residues were conserved in the M protein of KU viruses (75.9% conserved sites). Some KU viruses contained amino acid mutations in previously identified B-cell and T-cell epitopes [12, 41, 43]. Interestingly, amino acid substitutions were found in residues at positions 10 and 70, both of which are involved in susceptibility to virus neutralization by swine polyclonal antibodies [16, 36]. The residue at position 10 of

Table 3 Selection pressure profiles of Korean PRRSV-2 identified between 2012 and 2017

Structural protein	Mean dN/dS	Positively selected sites	
		N	Amino acid position (based on VR-2332)
GP2	0.230	21	2, 6, 7, 8, 9, 12, 13, 16, 17, 21, 22, 32, 39, **45**[a], 118, 238, 240, 250, 252, 254, 256
E	0.219	2	63, 64
GP3	0.290	36	2, 3, 6, 7, 8, 9, 11, 12, 13, 15, 18, 19, 21, 22, 23, 25, 27, 28, 30, 32, 153, 205, 206, 207, 211, 213, 215, 218, 222, 223, 225, 228, 233, 248, 250, 251
GP4	0.189	13	2, 4, 5, 6, 12, 13, 14, 15, 46, **57**[a], **59**[a], **61**[a], **63**[a]
GP5	0.277	13	13[a], 14[a], 15[a], 19, 24, 25, 26, 30[b], 33[b], 34[b], 35[b], 189[a], 192[a]
ORF5a	0.346	4	41, 42, 43, 46
M	0.110	2	66[c], 128
N	0.204	1	5

[a]Sites of previously identified B-cell epitopes. Bold indicates sites that correspond to neutralizing epitopes of PRRSV-1

[b]Potential glycosylation sites

[c]Sites for previously identified T-cell epitopes

four viruses was tyrosine, which is associated with susceptibility to virus neutralization (Fig. 3B). For the residue at position 70, 25 viruses exhibited antibody escape variants with lysine. All epitopes of the M protein were conserved among KU viruses. However, some amino acid substitutions were found at positions 10, 13, 19, 62, 66, and 70 within two T-cell epitopes, and 164 within a B-cell epitope (Supplementary Figures).

Because the N protein is highly immunogenic in pigs and mice, extensive epitope mapping has been conducted; this resulted in more than 80% of the region being identified as antigenic [1, 12, 42, 45]. In this study, only epitopes that were commonly identified in two different studies were considered overlapping epitopes of the N protein; specifically, these were aa 11–25, 41–55, and 79–87, and these regions were subsequently analyzed. In KU viruses, the B-cell epitope region (aa 41–55) exhibited diverse mutations, especially at position 41 to 49 (Supplementary Figures).

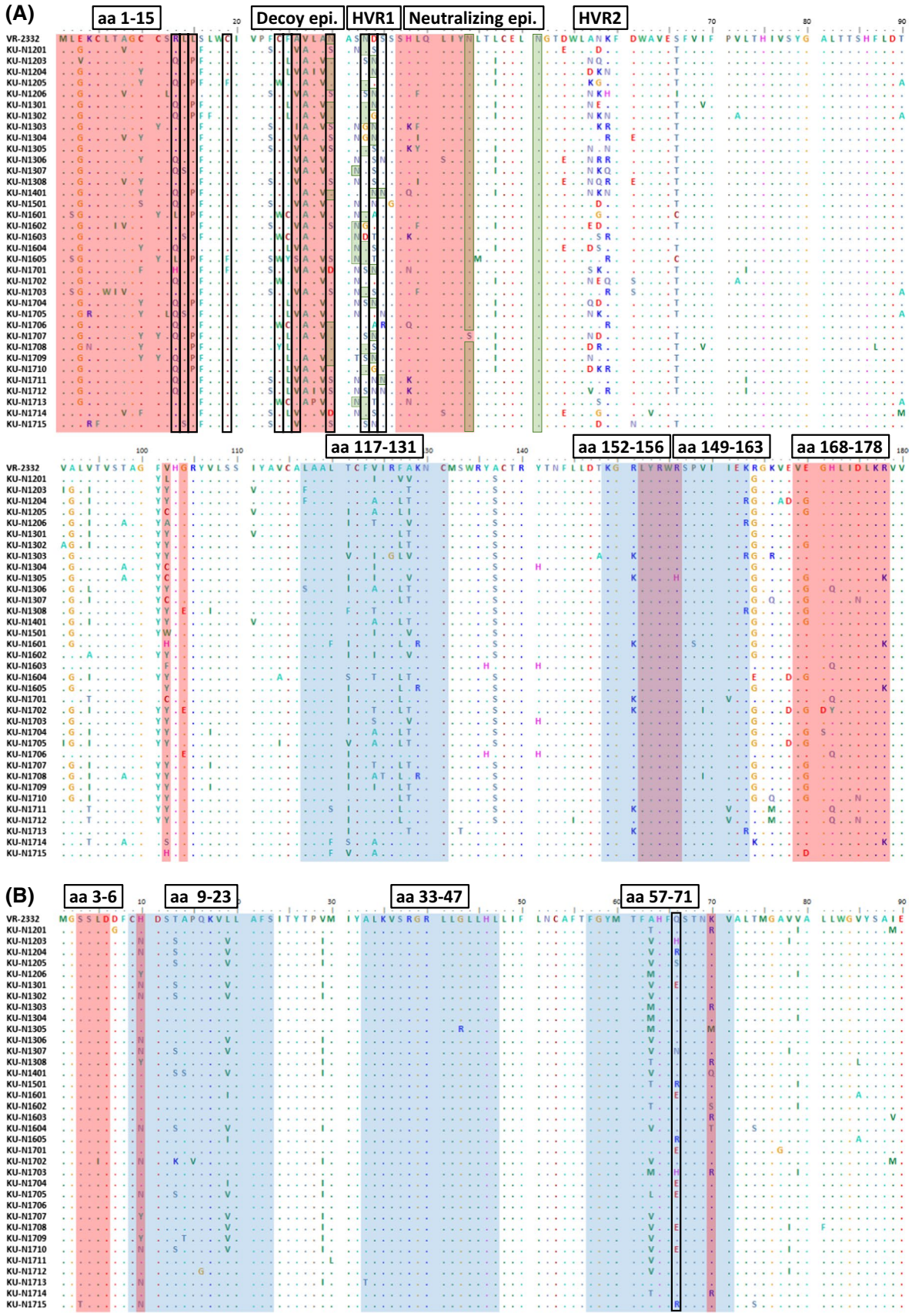
Minor structural proteins (GP2, GP3, and GP4) of Korean PRRSV-2

For GP2, two linear B-cell epitopes have been identified (aa 41–55 and aa 121–135) [12]. Of them, epitope 41–55, was variable among KU viruses, and the residue at position 45 was determined to have evolved under positive selection (Fig. 4A). The glycosylation patterns of all KU viruses were conserved at positions 178 and 184. GP3 of PRRSV-2 consists of four consecutively overlapping B-cell epitopes, aa 61–75, aa 71–85, aa 81–95, and aa 91–105 [12]. As the second most variable structural protein among KU viruses after GP5, a high level of variation was identified in the N- and C-termini of GP3. In contrast, each epitope was slightly variable with ten, ten, nine, and seven substitution sites within each consecutive epitope, respectively (Supplementary Figures). The prediction of putative N-glycosylation indicated that whereas N42, N50, N131, N160, and N195 were conserved among all KU viruses, thirteen and three KU viruses were N-glycosylation deletion and insertion variants at position 29 and 70, respectively, when compared to VR-2332. Especially, N70 was involved in a previously identified B-cell epitope, aa 61–75 (Fig. 4B). KU viruses showed a high level of variability within the B-cell epitope at positions 51 to 65. Especially, many amino acid substitutions with four positively selected sites were observed at positions 56 to 63 (Fig. 4C). Another interesting point was that a novel putative glycosylation site emerged at position 57 (N57) within the GP4 epitope of KU viruses.

Discussion

Since its first emergence on two different continents, PRRSV has become an important virus that causes enormous economic losses for the swine industry worldwide. Despite tremendous efforts to control and prevent PRRSV infection, this virus has rapidly spread worldwide and has become endemic to most pig-producing countries [27]. PRRSV has continued to evolve at a rate of $4.7–9.8 \times 10^{-2}$/sites/year, which is the highest rate among RNA viruses [20]. The rapid evolution of PRRSV contributes to the expansion of its genetic diversity and the emergence of new viral phenotypes worldwide. Under these circumstances, it is crucial to understand the genetic evolution of currently circulating PRRSV to introduce effective strategies to deal with PRRSV infection, such as the preparation of an effective and safe vaccine. To date, ORF5 of both PRRSV-1 and PRRSV-2 has been the main target of most molecular epidemiology studies because it is considered to have the highest sequence diversity among the structural ORFs [22, 26]. However, a small portion of the complete PRRSV genome cannot provide a clear representation of the genetic relationships and the evolution of PRRSV. In addition, the discovery of novel neutralizing epitopes has revealed the importance of minor structural proteins in the mechanism of immune evasion of PRRSV [38]. Therefore, this study investigated the genetic and evolutionary characteristics of whole structural genes of recently circulating PRRSV-2 strains in Korea.

Since its first isolation in 1994, PRRSV-2 has continuously evolved in the Korean pig population. Previous studies have demonstrated the independent evolution and high genetic variation of Korean PRRSV-2, based on the ORF5 region [5, 6]. Our study provides evidence of the continuous circulation of genetically diverse viruses in Korea with dynamics toward independent evolution. With a relatively high genetic difference of approximately 10%, the recently identified viruses formed unique Korean clades and had no phylogenetic relationship to PRRSV from other countries based on the ORF2–7-based tree. Korean field clade IV consisted of viruses that originated from Jeju Island, which is 80 km away and separated by the sea from the nearest pig farming regions in mainland Korea. PRRSV-2, which was

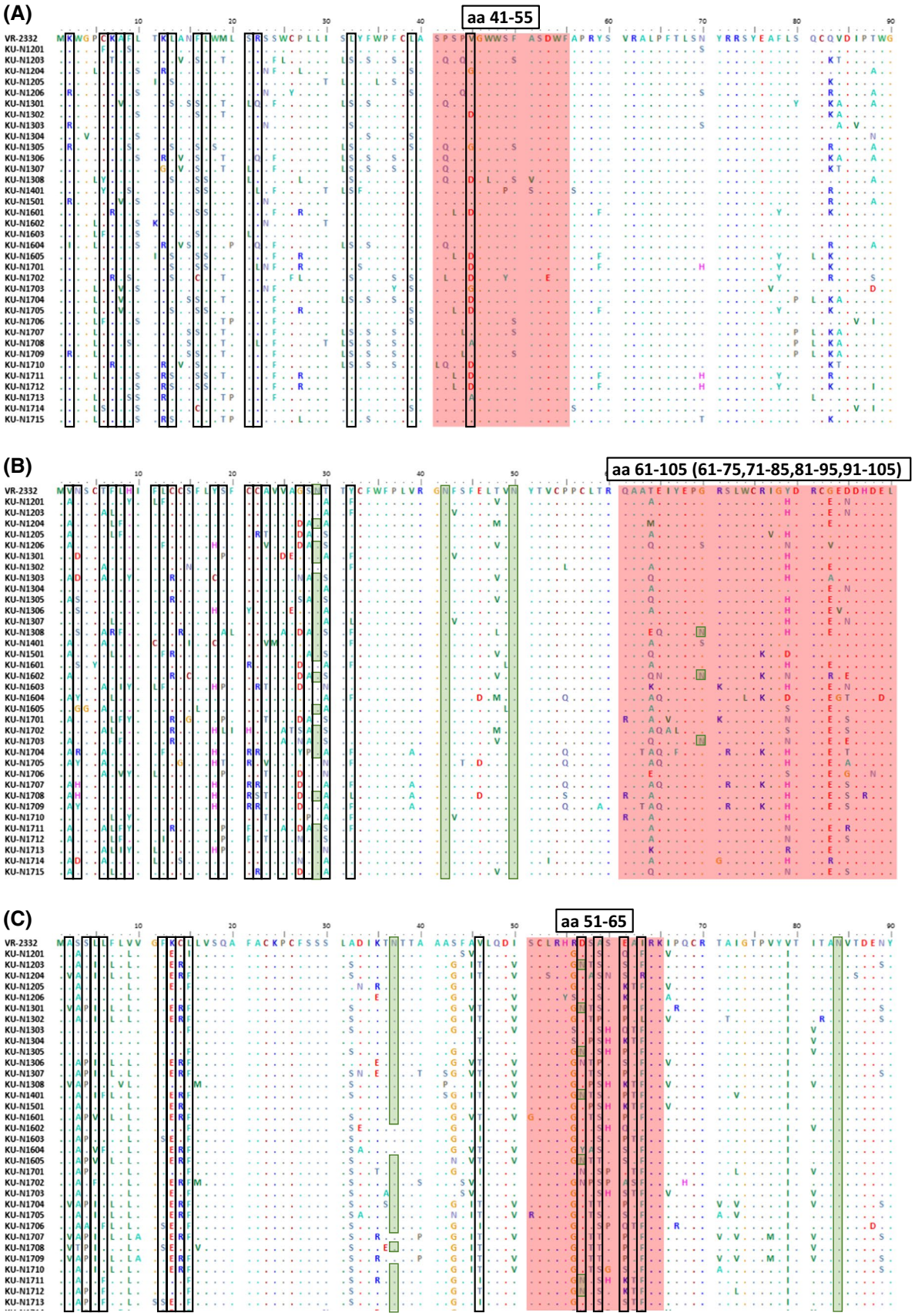

(A)
aa 1-15
Decoy epi.
HVR1
Neutralizing epi.
HVR2
aa 117-131
aa 152-156
aa 149-163
aa 168-178
(B)
aa 3-6
aa 9-23
aa 33-47
aa 57-71

◂**Fig. 3** Alignment of amino acid sequences of structural proteins of KU viruses. (A) GP5. (B) M. Red shade, previously identified B-cell epitope; blue shade, previously identified T-cell epitope; black box, positively selected sites; green shade, potential glycosylation site

found to belong to the Korean field clades, has been identified since the early to mid-2000s. These results indicate that Korean PRRSV-2 has evolved independently for the past decade and that geographical restriction has contributed to differences in viral evolution between Jeju Island and mainland Korea. In addition, our study shows that a novel NADC30-like virus had recently emerged in Korea. The introduction of new viruses increases the complexity of PRRSV-2 epidemiology in Korea. In China, the emergence of NADC30-like virus has received attention because of its high incidence of recombination [34]. Although no recombination events were detected in KU-N1702 in this study, we need to monitor the circulation of Korean NADC30-like PRRSV as well as other Korean PRRSV-2 strains in the field. The independent evolution of Korean PRRSV-2 was also demonstrated using an ORF5-based tree. However, the displacement of two viruses that belonged to clade I and the lack of a relationship between clades II and III and other lineages indicated that the ORF5-based tree could not fully represent the molecular epidemiology of Korean PRRSV-2. Further study with increased sample sizes and elaborate phylogenetic analysis would help to clarify the evolutionary epidemiology of Korean PRRSV-2. Currently, PRRSV circulates widely among Korean pig populations, and many infected farms have used modified live vaccines to control PRRSV infection. However, most vaccines used in Korea were made by international companies, and the viruses used originated in other countries. Under these circumstances, PRRSV-2 became "Koreanized" through independent evolution, and a novel NADC30-like strain emerged. The vast genetic heterogeneity between vaccine and field viruses dampens the efficacy of the vaccine. Although commercial vaccines are still effective for reducing the impact of PRRSV infection, they do not provide sufficient protection against genetically diverse PRRSV. Therefore, it is important to develop more-suitable vaccines to achieve a satisfactory level of protection against Korean PRRSV-2.

The major structural proteins of PRRSV, GP5 and M, form a heterodimer that interacts with host cell receptors and mediates viral penetration into the cell. This has motivated investigations aimed at determining the locations of neutralizing epitopes on the major structural proteins [29, 47]. These studies have resulted in the discovery of a linear neutralizing epitope in GP5 as well as the key residues that are involved in viral neutralization in GP5 and M protein [15, 16, 28, 36]. Consistent with previous findings, a linear neutralizing epitope was found to be conserved among KU viruses despite the high level of genetic diversity in the decoy epitope. However, a recently identified Korean PRRSV-2 isolate was found to have genetic mutations in key residues for viral neutralization, resulting in the circulation of viruses in the field that contain amino acids that are associated with a neutralizing-antibody-resistant phenotype. Although the ability of KU viruses to escape from neutralization was not investigated in this study, it is possible for these viruses to survive longer in infected pigs. This is because these mutations were found during *in vitro* selection using homologous polyclonal swine serum. Further study will evaluate the relationship between genetic mutations and resistance to neutralizing antibodies.

Despite continuous investigation of the function of minor structural proteins in viral neutralization, our knowledge about the evolution of these proteins is limited. In this study, our results demonstrated the genetic diversity of minor structural proteins of Korean PRRSV-2. Although most of the nucleotide substitutions were found in the N- and C-termini of the minor structural proteins, previously identified B-cell epitopes also were variable, including aa 41–55 of GP2, aa 61–75, aa 71–85, aa 81–95 of GP3, and aa 51–65 of GP4. Especially, it was revealed that the corresponding regions of three epitopes (aa 41–55 of GP2, aa 61–75 of GP3, and aa 51–65 of GP4) were targets for virus-neutralizing antibodies in PRRSV-1 [38]. Antibodies specific for these epitopes were shown to reduce the replication of PRRSV-1 virus in PAM in a dose-dependent manner. Surprisingly, we observed not only genetic variation in the neutralizing epitopes but also positively selected sites and the emergence of novel glycosylation sites within the epitopes. Viral evolution is directed by random mutation and natural selection. Through the process of natural selection, viruses that carry advantageous mutations that increase their fitness survive longer and eventually spread more to new host populations. Thus, estimating selection pressure provides important clues regarding the functional relevance of certain motifs. In PRRSV, positively selected sites were later identified as key residues that regulate susceptibility to viral neutralization [13, 15]. Furthermore, previous studies have indicated that N-linked glycosylation of PRRSV plays a critical role in virus infectivity, antigenicity, and the ability to induce neutralizing antibodies [2, 11, 40]. The absence of N34 and/or N51 in GP5 and


(A)
aa 41-55
(B)
aa 61-105 (61-75,71-85,81-95,91-105)
(C)
aa 51-65

◂**Fig. 4** Alignment of amino acid sequences of structural proteins of KU viruses. (A) GP2. (B) GP3. (C) GP4. Red shade, previously identified B-cell epitope; black box, positively selected sites; green shade, potential glycosylation site

N131 in GP3 has been shown to result in the induction of significantly high levels of neutralizing antibody titers in pigs, and this has been attributed to glycan shielding against immune evasion. In this respect, diversifying evolution and emergence of novel glycosylation sites within those regions are meaningful because they would allow the virus to evade the host immune system. Therefore, the direct effect of these changes on immune evasion should be further evaluated.

In summary, this study has broadened our understanding of the molecular epidemiology and genetic characteristics of currently circulating PRRSV-2 strains, based on the analysis of ORF2 to ORF7. Field strains of PRRSV-2 evolved independently in Korea with a high level of genetic variation and formed the distinct clades in ORF2–7 phylogeny. The emergence of an NADC30-like strain contributes to the expansion of genetic diversity, but no epidemiological evidence of its introduction route has been presented. While a neutralizing epitope in GP5 was conserved, KU viruses displayed genetic variation within the minor structural proteins. Especially, GP2 and GP4 neutralizing epitopes have evolved under positive selection, and new putative glycosylation sites have emerged in GP3 and GP4 neutralizing epitopes. Therefore, enhanced surveillance of PRRSV-2 and comprehensive studies on various regions of PRRSV are needed to assess viral evolution, which would provide novel insights for the development of effective strategies to combat this virus.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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