

Dynamics and evolution of porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 following modified live PRRSV vaccination in a PRRSV-infected herd

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Abstract The objective of this study was to investigate the dynamics and evolution of porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 following the use of a modified live PRRSV (MLV) vaccine. A PRRSV-positive farm with coexistence of types 1 and 2 and no history of MLV vaccination was investigated. Vaccination with a type 2 MLV (Ingelvac PRRS MLV, Boehringer Ingelheim, USA) was implemented. All sows were vaccinated at monthly intervals for two consecutive months and then every third month. Piglets were vaccinated once at 7–10 days of age and weaned to nursery facilities at 21–23 days of age. Serum samples were collected monthly before and after vaccination from four population groups, including replacement gilts and suckling, nursery and finishing pigs, and assayed by PCR. After a year of blood collection, amplified products were sequenced, resulting in 277 complete ORF5 gene sequences from 145 type 1 and 132 type 2 isolates. Prior to and following vaccination, both type 1 and type 2 PRRSV were isolated and found to coexist in an individual pig. Each genotype evolved separately without influencing the strain development of the other. Although the substitution rates of both genotypes were relatively similar, MLV vaccination appears to

increase the heterogeneity of type 2 PRRSV, resulting in the emergence of three novel type 2 PRRSV clusters in the herd, including an MLV-like cluster, which disappeared within the month following whole-herd vaccination. Two additional clusters included one related to the MLV vaccine and one related to the endemic cluster of the herd.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of a syndrome characterized by reproductive failure and respiratory disorders in pigs and has had a significant economic impact on the swine industry worldwide since its emergence in the late 1980s. PRRSV is an enveloped, positive-sense single-stranded RNA virus belonging to the family *Arteriviridae* in the order *Nidovirales*. Its genome is approximately 15 kb in length. Ten open reading frames (ORFs), designated as ORFs 1–7, have been identified [9, 17, 29]. Open reading frame 1 encodes the viral RNA polymerase and is divided into ORFs 1a and 1b, and this ORF comprises approximately 80 % of the genome [4]. The other six ORFs (ORFs 2–7) encode structural proteins, including glycoproteins (GP) 2–5, and the M and N proteins. Two additional structural proteins, E and ORF 5a, were discovered relatively recently [9, 29]. The ORF5 genes of PRRSV, playing important roles in genetic variation and protection, encode glycoprotein 5 (GP5) and is associated with neutralizing epitopes.

Two distinct genotypes of PRRSV, European (EU), or type 1, and North American (NA), or type 2, have been recognized, and the genetics of both genotypes are markedly different. PRRSV has quasispecies characteristics, resulting in genetic differences among isolates. The two

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genotypes of PRRSV have been described as evolving independently on different continents [18]. However, their coexistence has been increasingly evident in several countries, including Thailand, China, and Korea over the last few years [3, 12, 15, 19, 26], raising questions concerning clinical severity, genetic diversity, and control methods involving vaccination of herds co-infected with both genotypes.

PRRSV control in Thailand has been accomplished through acclimatization of replacement stocks prior to introduction into the herd. In addition, several vaccination protocols with modified live virus (MLV) vaccines have been implemented in swine farms including vaccination of whole sow herds every 2–3 months, vaccination of piglets at 7–10 days of age, and pre-farrow vaccination. At present, two MLV PRRSV vaccines representing types 1 and 2 are commercially available in Thailand. However, PRRSV control practices using vaccination with a type 2 strain have been used preferentially because the manufacturer claims that the type 2 MLV vaccine provides cross-protection against challenge of both type 1 and 2 (http://bi-vetmedica.com/sites/default/files/ingelvac_PRRS_MLV_rp.pdf). Although MLV vaccination has been used, many well-vaccinated herds have experienced sporadic disease outbreaks of both reproductive and respiratory diseases.

Both genotypes of PRRSV are endemic in most Thai swine herds. Questions remain as to how MLV vaccination with only the type 2 strain influences the genetic diversity of both genotypes of PRRSV in co-infected herds. Genetic variation of PRRSV is commonly observed in the same herd, and progeny viruses differ from their parent viruses by 0.47 %–1 % and 0.71 %–1.99 % in the nucleotide and amino acid sequence, respectively, of the ORF5 gene [7]. The continuous evolution and emergence of new variant isolates could potentially cause a small outbreak. The influence of MLV vaccination on genetic diversity remains unknown. Therefore, the objective of this study was to investigate the genetic evolution of PRRSV following MLV vaccination in a chronically PRRSV-infected herd. The results reported herein provide insight into the genetic evolution of coexisting PRRSV genotypes under the influence of MLV vaccination. This knowledge will facilitate the design and implementation of a more successful PRRSV prevention and control program.

Materials and methods

Herd information

The study was conducted in a swine herd with no history of MLV vaccine use. The herd had an inventory of

1,700 sows and operated with a one-site farrow-to-finish production facility (Supplementary Material 1). The breeding herd had six buildings designated for breeding, gestation, and farrowing. Half of each building was designated for breeding and gestating activities, and the other half was for farrowing activity. The farrowing facilities operated all-in/all-out by week and allow a week of downtime. All sows were artificially inseminated on-site using PRRSV-negative semen from PRRSV-free boars. The farm had its own boar stud located 5 km away from the breeding herd. All boars in the stud were PRRSV free. Semen was tested by PCR prior to insemination.

A parity segregation system was used in which one building was designated for breeding, gestating, and farrowing of primiparous sows. Following weaning, first-parity sows were moved as a replacement to other multiparous sow facilities. Pigs were weaned at 21–23 days of age and moved to nursery facilities. Four nursery facilities were adjacent to the breeding facilities, and each building was divided into two halves. Each half of the building operated all-in/all-out by week. Nursery pigs were moved at approximately nine weeks of age to finishing facilities located 30 m away.

Replacement gilts were internally produced and housed with nursery and finishing pigs. They were moved to acclimatization facilities located in finishing facilities at 18 weeks of age and introduced to the breeding herd at 32–33 weeks of age.

Prior to MLV vaccination, the studied herd was positive for both type 1 and type 2 PRRSV. Type 2 MLV (Ingelvac PRRS MLV, Boehringer Ingelheim, USA) vaccination was implemented in January 2010. All sows were repeatedly vaccinated at monthly intervals for two consecutive months, followed by vaccination every third month. Piglets were vaccinated once at 7–10 days of age and moved to nursery facilities at 21–23 days of age. Replacement gilts were vaccinated with two doses of type 2 MLV at 18 and 22 weeks of age. No change in other management strategies was observed.

Experimental design

The study was conducted from November 2009 to December 2010. Serum samples were collected monthly for three consecutive months prior to vaccination and another six consecutive months, and then twice at a bimonthly interval after the second vaccination. At each sampling time, five samples were taken from each four population groups, including replacement gilts, suckling pigs, nursery pigs, and finishing pigs. Sera were assayed for the presence of viruses by PCR.

PCR and sequence determination

Total RNA was extracted from serum samples using a Nucleospin[®]RNA Virus Kit (Macherey-Nagel, Germany) in accordance with the manufacturer's instructions. cDNA was synthesized from the extracted RNA using M-MuLV reverse transcriptase (New England BioLabs Inc., MA, USA).

PCR amplification was performed on the cDNA. To amplify type 2 progeny viruses, primers ORF5 USF (5' - CCT GAG ACC ATG AGG TGG G - 3') and ORF5 USR (5' - TTT AGG GCA TAT ATC ATC ACT GG - 3') were used, and PCR amplification was performed using GoTaq[®]Green Master Mix (Promega, USA). After an initial incubation at 95 °C for 2 min, the reaction was subjected to 35 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s, followed by a final five-minute extension at 72 °C.

To amplify type 1 progeny viruses, primers ORF5 EUF (5' - TGA GGT GGG CTA CAA CCA TT - 3') and ORF5 EUR (5' - AGG CTA GCA CGA GCT TTT GT - 3') were used, and PCR amplification was performed using GoTaq[®]Green Master Mix (Promega, USA). After an initial incubation at 94 °C for 4 min, the reaction was subjected to 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, followed by a final five-minute extension at 72 °C.

Amplified PCR products were purified using a PCR purification kit (Macherey-Nagel, Germany). Sequence reactions were performed at Biobasic Inc. (Ontario, Canada) using an ABI Prism 3730XL DNA sequencer.

Sequence analysis

Nucleotide sequences of ORF5 genes were aligned using CLUSTALW [27]; amino acid sequences were aligned using BioEdit. ORF5 sequences were analyzed for the presence of recombination events using Recombination Detection Program [16] and were further analyzed for potential recombination breakpoints using the Genetic Algorithm for Recombination Detection (GARD) [13].

Neighbor-joining (NJ) trees were generated with a Kimura 2-parameter model using MEGA 5 [25]. The robustness of the phylogenetic analysis and significance of the branch order were determined by bootstrap analysis with 1000 replicates. Bayesian MCMC analysis for estimating the substitution rate and time to the most recent common ancestor of post-vaccination isolates were performed using BEAST v.1.6.2 [5].

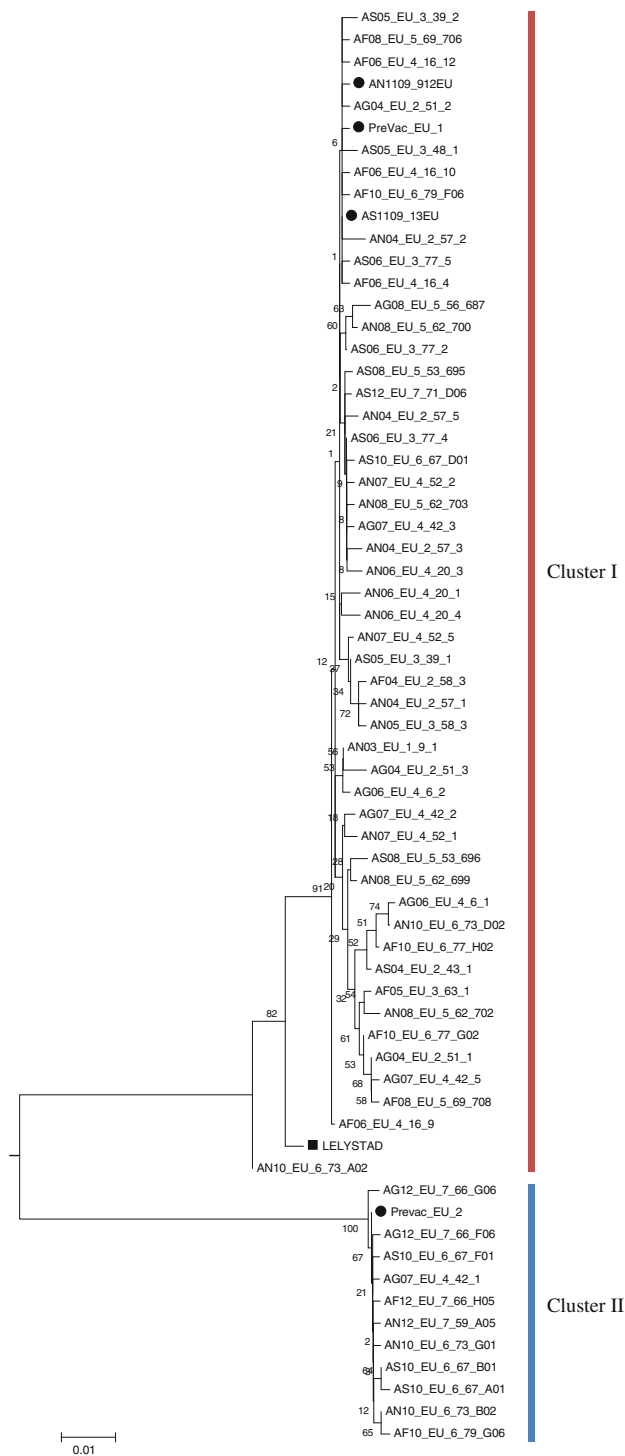
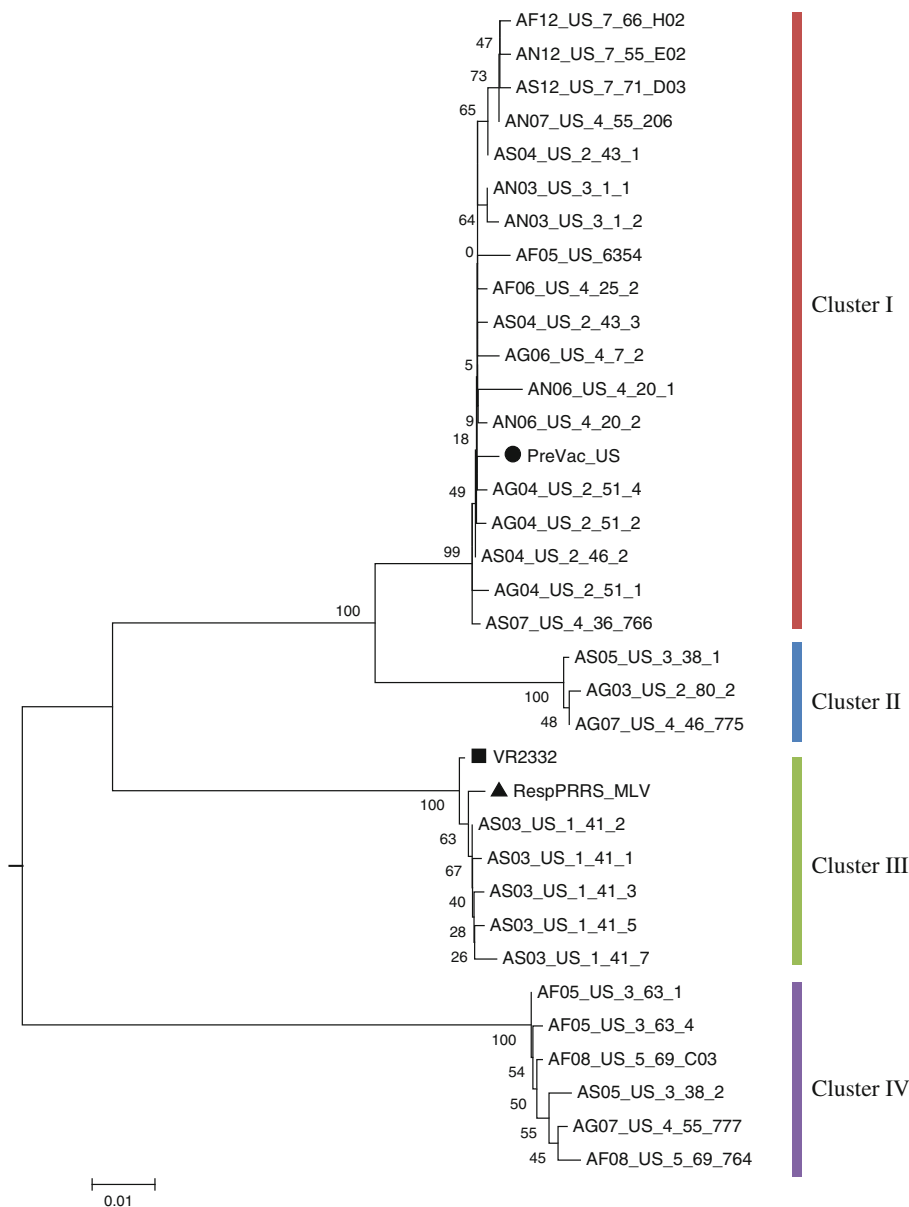


Fig. 1 Neighbor-joining tree of genotype 1 PRRSV isolates based on the nucleotide sequences of complete ORF5 genes. Filled circles represent the pre-vaccination isolates. Filled rectangles represent the genotype 1 prototype virus (Lelystad virus). The rest of the sequences are from post-vaccination isolates

Fig. 2 Neighbor-joining tree of genotype 2 PRRSV isolates based on the nucleotide sequences of complete ORF5 genes. Filled circles represent the pre-vaccination isolates. Filled rectangles and triangles represent the genotype 2 prototype virus (VR-2332) and the modified live PRRSV vaccine of genotype 2, respectively. The rest of the sequences are from post-vaccination isolates



Results

Phylogenetic analysis

A total of 277 complete ORF5 sequences of progeny PRRSV were obtained from the herd during a year of collection. One hundred forty-five sequences belonged to the type 1 isolates, while the other 132 belonged to type 2. Recombination events between the two genotypes and between isolates within type 2 were not evident. Identical ORF5 sequences found at the same or different sampling times were identified and excluded, resulting in 63 type 1 and 33 type 2 unique sequences for further genetic analysis. These non-redundant ORF5 sequences have been deposited in GenBank under accession numbers JQ040720–JQ040771 (type 1 isolates)

and JQ040772–JQ040797 (type 2 isolates). Four type 1 (accession numbers KCI174330–KCI174333) and one type 2 unique ORF5 sequences (accession numbers KCI174334) were identified from the isolates collected during the three months before vaccination and used as pre-vaccination reference sequences in the phylogenetic analysis.

To investigate the genetic relationship of these progeny viruses, a phylogenetic analysis was performed on the non-redundant ORF5 sequences of PRRSV isolated after vaccination, the isolates endemic to the herd prior to vaccination, the type 2 prototype virus (VR-2332; accession number U87392) and its derived vaccine virus (Ingelvac PRRS MLV, Boehringer Ingelheim, USA) Accession Number AF066183, and the type 1 prototype virus (LV; accession number M96262). The resulting NJ tree suggested the independent

evolution of each PRRSV genotype. The phylogenetic tree demonstrated that the type 1 isolates were separated into two clusters (Fig. 1). Pairwise nucleotide and amino acid identity values between the clusters were in the range of 85.9 %-88.4 % and 84.5 %-90.0 %, respectively. Cluster I contained 53 isolates, including LV and three isolates identified in the herd prior to vaccination. Cluster II included one pre-

vaccination isolate along with 11 isolates identified following vaccination. It is noteworthy that three ORF5 sequences, two in cluster I and one in cluster II, identified after vaccination, were identical to isolates that had been detected previously in the herd.

In contrast to the type 1 isolates, the type 2 isolates were grouped into four clusters (Fig. 2). Cluster I included the

Fig. 3 The distribution of sequence cluster size throughout the year of post-vaccination collection. The number of isolates in each cluster is displayed for each sampling month. **a** genotype 1; **b** genotype 2. The cluster assignments are based on phylogenetic analysis

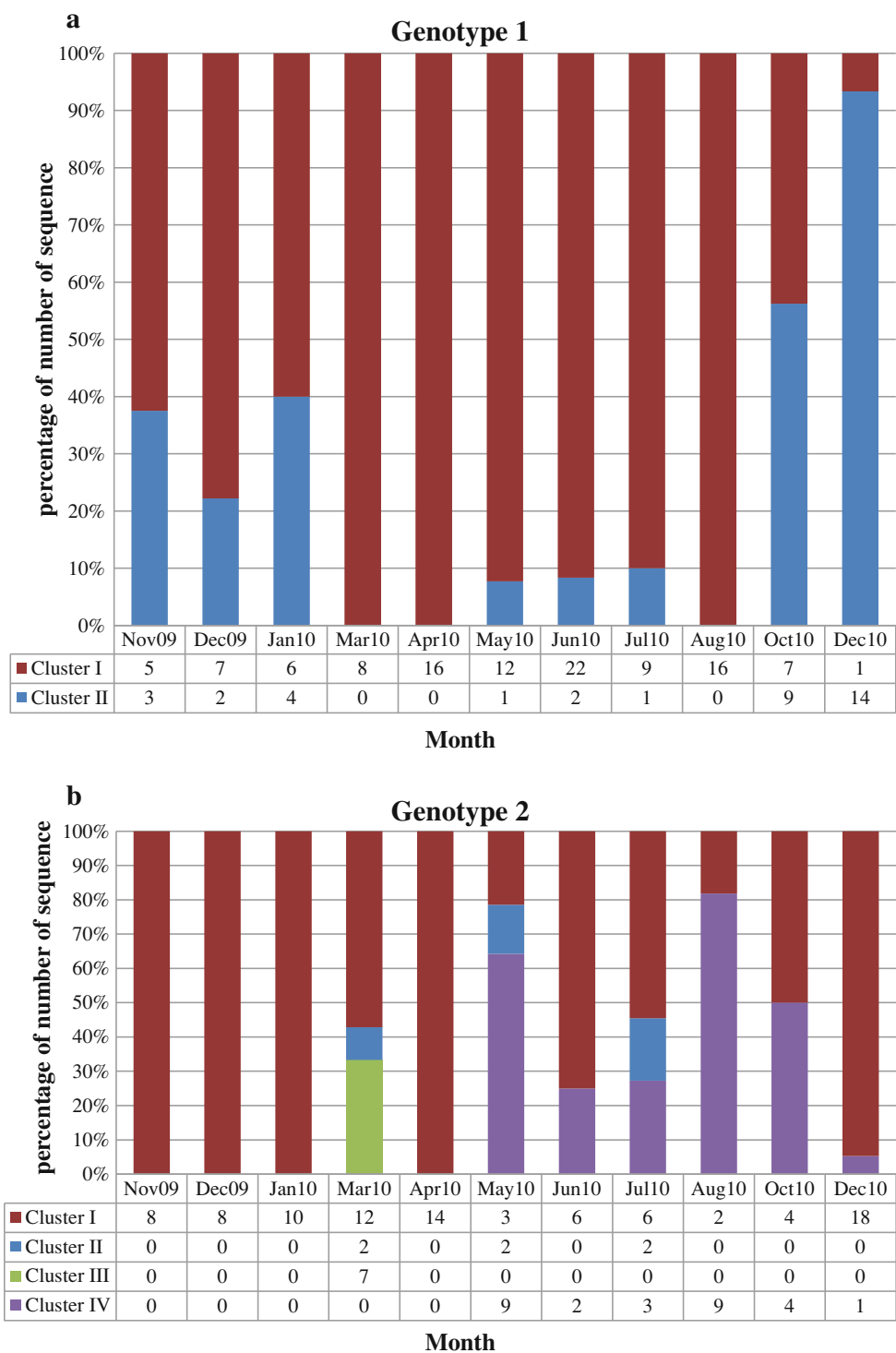


Table 1 Pattern of deduced amino acid positions 27-45 and number of ORF5 genes of genotype 2 in each cluster by month after the first vaccination

Cluster	Deduced amino acid position 27-45**	Months after the first vaccination								
		3	4	5	6	7	8	10	12	
I	VLANASNTNSSHFQLIYNL	12	14	3	6	6	2	4	18	
II	VLVDANNTSSSHFQLIYNL	2 ^{S*}	-	2 ^N	-	2 ^{G,S}	-	-	-	
III	VLANASNDSSSHLQLIYNL	7 ^S	-	-	-	-	-	-	-	
IV	VLVSASNNSSSHLQLIYNL	-	-	-	2 ^N	-	9 ^{S,F}	4 ^S	1 ^S	
IV	VLVSASNNSSSHLLLSYNL	-	-	9 ^S	-	-	-	-	-	
IV	VLVSASNNSSSHLLLS CNL	-	-	-	-	3 ^{S,N}	-	-	-	

* Superscripts indicate population groups from which virus was isolated. The population groups are as follows: G, replacement gilts; S, suckling pigs; N, nursery pigs; and F, finishing pigs. No superscript means that virus was isolated from all population groups

** Decoy ((A/V)²⁷LVN) and primary neutralizing (S³⁷H(F/L)QLIYN) epitopes of PRRSV North American genotype

isolate identified prior to vaccination, which was also detected after vaccination, and 18 post-vaccination isolates. Clusters I and II had nucleotide and amino acid sequence identities of 94.8 %-95.3 % and 91.5 %-93.5 %, respectively. Cluster III included the type 2 prototype virus (VR-2332), its derived vaccine virus (Ingelvac PRRS MLV, Boehringer Ingelheim, USA), and five post-vaccination isolates. Interestingly, these five isolates, sharing 99.99 % nucleotide and amino acid sequence identity with the type 2 MLV, emerged a month after MLV vaccination and disappeared during the course of the study. Cluster IV contained isolates collected five months after vaccination and throughout the remainder of the study (Fig. 2).

Based on the phylogenetic analysis, the number of sequences from each sampling time that fall into each cluster is displayed in Fig. 3. All complete ORF5 sequences, including identical ones, were considered in this case. Although each cluster showed no obvious separation by sample collection time for the type 1 isolates, the proportion of sequences in cluster II versus cluster I seemed to grow with time. Most of the sequences in cluster II were isolated in months 10 (Oct 10) and 12 (Dec 10) (Fig. 3). The observed patterns for the type 2 isolates were the presence of isolates from cluster I throughout the study, the inconsistent presence of cluster II isolates, the emergence and disappearance of cluster III sequences in a short time period, and the rise of cluster IV sequences from month 5 onward (Fig. 3).

It is noteworthy that the emergence of the novel clusters III and IV was primarily observed in suckling pigs (Table 1).

Cluster characterization

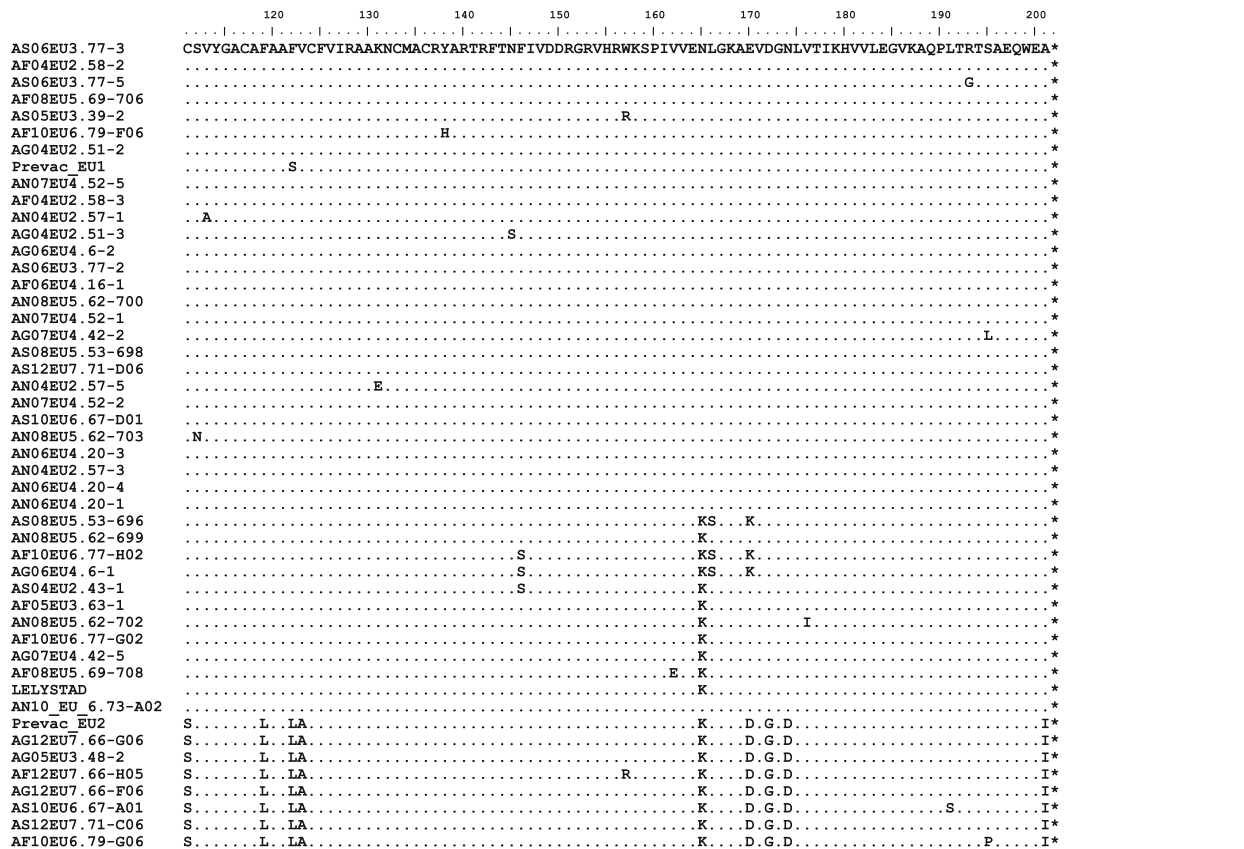
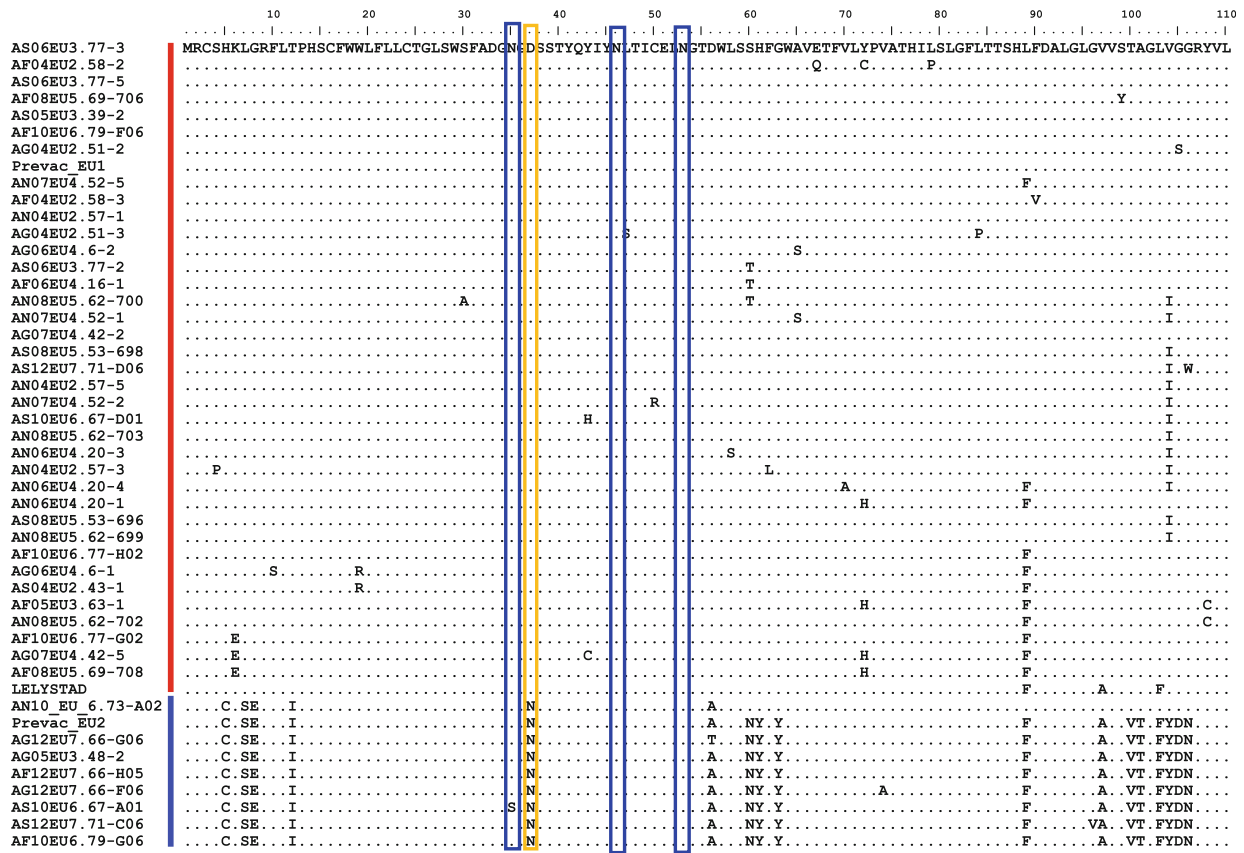
The deduced amino acid sequences were aligned separately for each genotype to investigate the amino acid sequence differences among isolate clusters. Important motifs in GP5, including previously described decoy and primary

Fig. 4 Alignment of glycoprotein 5 amino acid sequences from European isolates. The conserved glycosylation sites are highlighted in blue boxes, while non-conserved sites are in yellow boxes. Sequences of different clusters are labeled as bars of different colors: red for cluster I and light blue for cluster II (color figure online)

neutralizing epitopes (PNEs) [21–23] and potential glycosylation sites, were analyzed. Characterization of the clusters in both genotypes was based on the number and position of potential glycosylation sites. Both clusters of the type 1 isolates had three conserved glycosylation sites at positions 35, 46, and 53 (Fig. 4). Isolates in cluster II had one additional glycosylation site at position 37. In contrast, the type 2 isolates had three conserved glycosylation sites at positions 33, 44, and 51 (Fig. 5). The variation was observed mainly in the positions between decoy and neutralizing epitopes. Isolates in clusters I through IV had an additional 2-3 glycosylation sites at amino acid positions 30-35 (³⁰NASNTN³⁵, ³⁰DANNTS³⁵, ³⁰NASNDS³⁵, and ³⁰SASNNS³⁵).

Bayesian analysis of post-vaccination PRRSV

To estimate the substitution rate and divergence time of each PRRSV genotype under vaccine selective pressure, we used Bayesian MCMC analysis to analyze the ORF5 sequences given the exact collection times. Identical sequences identified in the same month were excluded from the analysis. Bayes factors were used for model comparison to determine which evolutionary model yielded the best results. Since the calculated Bayes factors suggested that the HKY substitution model fit the data better than the GTR model, we did the analysis using the HKY model with a range of component parameters. The mean time to the most recent common ancestor (TMRCA), the substitution rate, and the 95 % highest posterior probability density (HPD) are displayed in Table 2 for 12



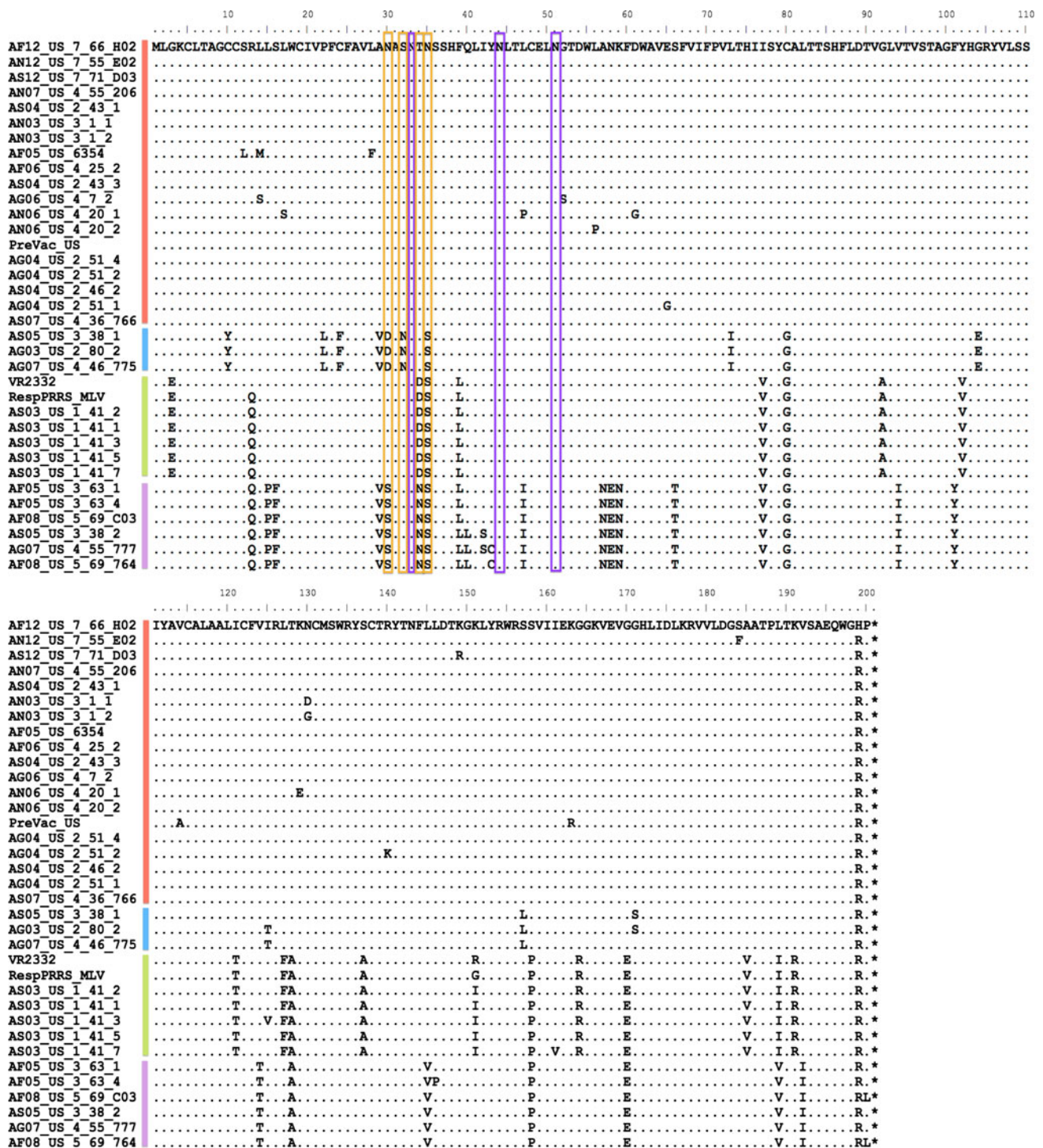


Fig. 5 Alignment of glycoprotein 5 amino acid sequences from genotype 2 isolates. The conserved glycosylation sites are highlighted in purple boxes, while non-conserved sites are in orange boxes.

Sequences of different clusters are labeled as bars of different colors: red for cluster I, light blue for cluster II, light green for cluster III, and light purple for cluster IV (color figure online)

different evolutionary models. According to the Bayes factor values, the best model found for both type 1 and type 2 was the uncorrelated exponential relaxed-clock model with a constant-size coalescent tree prior and no rate

heterogeneity among sites. Under this evolutionary model, the rate of substitution was estimated to be 5.02×10^{-4} per site per month for the type 1 virus and 6.30×10^{-4} per site per month for type 2.

Table 2 The calculated substitution rate and TMRCA for PRRSV ORF5 sequences collected after the vaccination period

Model	TMRCA (months before)		Substitution rate (/site/month)	
	Mean	95 %HPD	Mean	95 %HPD
(A) Genotype 1				
HKY + none + strict	198.87	109.22–309.68	4.01×10^{-4}	2.23×10^{-4} – 5.92×10^{-4}
HKY + none + relaxed (lognormal)	181.53	64.24–321.25	4.31×10^{-4}	2.31×10^{-4} – 6.47×10^{-4}
HKY + none + relaxed (exponential)*	147.29	41.45–291.59	5.02×10^{-4}	2.62×10^{-4} – 7.56×10^{-4}
HKY + Γ + strict	197.23	112.49–297.92	4.04×10^{-4}	2.40×10^{-4} – 5.84×10^{-4}
HKY + Γ + relaxed (lognormal)	180.38	73.42–318.51	4.35×10^{-4}	2.38×10^{-4} – 6.48×10^{-4}
HKY + Γ + relaxed (exponential)	146.22	42.29–292.67	5.11×10^{-4}	2.56×10^{-4} – 7.79×10^{-4}
HKY + I + strict	206.97	113.08–316.61	4.12×10^{-4}	2.33×10^{-4} – 6.01×10^{-4}
HKY + I + relaxed (lognormal)	188.10	72.04–328.21	4.35×10^{-4}	2.38×10^{-4} – 6.48×10^{-4}
HKY + I + relaxed (exponential)	152.52	44.86–303.11	5.04×10^{-4}	2.53×10^{-4} – 7.77×10^{-4}
HKY + Γ + I + strict	208.84	115.86–324.77	4.10×10^{-4}	2.32×10^{-4} – 6.04×10^{-4}
HKY + Γ + I + relaxed (lognormal)	186.05	68.97–314.92	4.41×10^{-4}	2.40×10^{-4} – 6.75×10^{-4}
HKY + Γ + I + relaxed (exponential)	160.51	43.67–322.63	4.98×10^{-4}	2.41×10^{-4} – 7.69×10^{-4}
(B) Genotype 2				
HKY + none + strict	244.05	102.02–442.67	4.02×10^{-4}	1.48×10^{-4} – 6.71×10^{-4}
HKY + none + relaxed (lognormal)	193.44	39.29–383.94	4.80×10^{-4}	1.59×10^{-4} – 8.75×10^{-4}
HKY + none + relaxed (exponential)*	115.38	35.68–224.13	6.30×10^{-4}	2.30×10^{-4} – 1.07×10^{-3}
HKY + Γ + strict	235.76	100.18–428.99	4.07×10^{-4}	1.58×10^{-4} – 6.85×10^{-4}
HKY + Γ + relaxed (lognormal)	195.96	46.26–385.84	4.61×10^{-4}	1.50×10^{-4} – 8.18×10^{-4}
HKY + Γ + relaxed (exponential)	112.80	36.18–225.46	6.26×10^{-4}	2.24×10^{-4} – 1.06×10^{-3}
HKY + I + strict	239.80	101.32–431.99	4.04×10^{-4}	1.51×10^{-4} – 6.64×10^{-4}
HKY + I + relaxed (lognormal)	198.85	49.04–397.43	4.65×10^{-4}	1.51×10^{-4} – 8.40×10^{-4}
HKY + I + relaxed (exponential)	118.41	37.47–241.34	6.29×10^{-4}	2.26×10^{-4} – 1.11×10^{-3}
HKY + Γ + strict	241.73	101.23–435.47	4.03×10^{-4}	1.55×10^{-4} – 6.74×10^{-4}
HKY + Γ + I + relaxed (lognormal)	198.46	44.72–390.23	4.66×10^{-4}	1.47×10^{-4} – 8.38×10^{-4}
HKY + Γ + I + relaxed (exponential)	112.31	37.76–220.75	6.36×10^{-4}	2.53×10^{-4} – 1.10×10^{-3}

The values of substitution rate and TMRCA were calculated using BEAST. These values were calculated based on the HKY substitution model with a coalescent constant-size tree prior. The among-site rate heterogeneity models used are no rate heterogeneity among sites (none), gamma-distributed rate heterogeneity (Γ), a proportion of invariant sites (I), and both gamma-distributed rate heterogeneity and invariant sites ($\Gamma + I$). Three models for rate variation among branches in the tree were used: the strict molecular clock model (strict), the uncorrelated lognormal relaxed-clock model (lognormal), and the uncorrelated exponential relaxed-clock model (exponential). The mean and 95 % highest posterior probability density (HPD) intervals of the Bayesian posterior estimates of substitution rate and TMRCA are shown for each evolution model. The chain length was adjusted for each model to make sure that all variables have an effective sample size (ESS) ≥ 200 . The asterisk indicates the best model according to the Bayes factor values

Discussion

Both type 1 and type 2 PRRSV concurrently exist in the Thai swine population with no preference for either type [19, 28]. Considering the co-infection phenomenon in the Thai swine herd, it has become a major question which type of MLV should be used for vaccination, given the availability of both types of MLV in Thailand. This study investigated the dynamics and evolution of PRRSV ORF5 following the use of the type 2 MLV in a PRRSV-infected herd.

Prior to MLV vaccination, both type 1 and type 2 PRRSV were long established in the studied herd and even coexisted in an individual pig, suggesting an endemic

status of both genotypes in the herd. Following vaccination, isolates of both genotypes evolved separately with approximately equal substitution rates. However, the mechanisms underlying the increased genetic diversity of these genotypes were different. The divergence of type 1 ORF5 sequences occurred through random substitution. In contrast, the mechanism utilized by type 2 to increase sequence variation was the addition of N-linked glycosylation sites in decoy epitopes (DCEs) and regions between decoy and primary neutralizing epitopes (PNEs), as evidenced by the emergence of three novel clusters after MLV vaccination: an ancestor-related group (cluster II), an MLV-related group (cluster III), and a novel group (cluster IV). However, increased diversity of type 2 isolates by the

emergence of novel clusters appeared following the introduction of MLV vaccination into the herd. This phenomenon was not observed prior to vaccination. These results suggested that the genetic diversity of the type 2 viruses in this herd was potentially under the influence of MLV. It can also be speculated that the lateral introduction of exotic isolates could also be responsible for the emergence of novel clusters in this herd. However, in this case, it is not clear why two clusters would be introduced concurrently. In addition, the herd was clinically normal, and there were no clinical signs of PRRSV-related disease throughout the study.

PRRSV is able to be persistently infective following an initial introduction. The variants in cluster I were isolates that had been endemic to the herd prior to vaccination. Cluster II likely evolved from cluster I, with only a few changes being observed in DCEs and positions between DCEs and PNEs. Interestingly, the MLV-related group (cluster III) lacked the ability to establish a persistent infection, as evidenced by its fading out within a month. One possible explanation for this is the small sample size. In addition, repeated vaccination with homologous isolates resulted in a shortened duration of the viremic phase [2, 8, 14]. This could result in reduced evidence of transplacental infection, which would cause these MLV-related isolates to be undetectable in piglets. Finally, MLV-related variants might have mutated at important amino acid positions, resulting in the emergence of novel isolates. Cluster IV was novel; however, the source of introduction was speculative. The long, persistent infection by isolates of this cluster was of interest. The presence of amino acid Q¹³ and sequence variation in regions described previously [1, 10, 20] suggested that these isolates were possibly derived from the MLV strain, with increased variation in N-linked glycosylation sites to evade the immune response and stay infective in the herd. However, it should not be concluded that the type 1 virus would not utilize a similar mechanism. This phenomenon might be explained by the lack of introduction of novel type 1 isolates into the herd during the study. In addition, whether the use of only type 1 MLV in herds co-infected with both types of viruses would result in the development of new clusters in Thailand remains to be studied.

The substitution rates of both types of viruses in this study were relatively similar. However, the substitution rate of the type 1 virus was higher than reported previously [6, 11]. In contrast, the substitution rate of the type 2 virus was similar to a previous report from China in which substitution increased the evolutionary rate after outbreaks [24]. The result of the high evolutionary rate of the type 2 virus in this study would suggest that the evolutionary rate is accelerated following the introduction of exotic isolates into the herd.

Breeding herd performance was improved after MLV vaccination. Although three novel type 2 clusters emerged following type 2 MLV vaccination, questions as to whether the new isolates would cause small outbreaks remain unanswered after a year of data collection in this study. Additional follow up on the evolution of PRRSV ORF5 and outbreaks in this herd in the near future is needed to answer those questions. In addition, a larger sample size in each population group is needed to increase the strength of the study.

In conclusion, MLV vaccination had no influence on strain development of other types of viruses. However, MLV vaccination influenced strain development in the same virus type and played a role in increased diversity of PRRSV ORF5 sequences in the herd by adding new clusters.

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