

# Dynamics and evolution of genotype 1 porcine reproductive and respiratory syndrome virus following its introduction into a herd concurrently infected with genotypes 1 and 2

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## Abstract

This study was conducted to investigate the dynamics and evolution of porcine reproductive and respiratory syndrome virus (PRRSV) genotype 1 following its introduction into a herd concurrently infected with both PRRSV genotypes. The results demonstrated that 2 novel genotype 1 groups emerged, termed clusters II and III. The emergence of cluster II suggested that this cluster of genotype 1 virus potentially correlated with the outbreak. Then, cluster III was detected with the disappearance of cluster II. The evolution of the genotype 1 virus from cluster II to cluster III is likely to be associated with immune evasion induced by amino acid substitutions at N-linked glycosylation sites. In contrast, evolution of the genotype 2 isolates was not observed. This result suggests that genotype 2 viruses were not affected by the emergence of the genotype 1 virus and each genotype developed independently.

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**Keywords:** Porcine, PRRSV, Genotype 1, Evolution, Thailand

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## Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive disorders in sows, including increased premature farrowing, a low farrowing rate, a high abortion rate, reduced numbers of weaned pigs, increased numbers of stillborn pigs, mummified fetuses and weak pigs, as well as respiratory disorders in nursery to finishing pigs (Collins *et al.*, 1992; Mengeling *et al.*, 1998). The PRRS is caused by the PRRS virus (PRRSV), an enveloped, positive-sense single-stranded RNA virus belonging to the family *Arteriviridae*, order *Nidovirales*. The PRRSV genome consists of ten open reading frames (ORFs) including ORFs 1-7. ORF1, comprising approximately 80% of the total genome, and is further divided into ORF1a and ORF1b, which encode non-structural proteins (nsp). The other ORFs (ORFs 2-7) encode six structural proteins, including glycoproteins (GP) 2-5 and the M and N proteins. Two additional structural proteins, E and ORF5a, have also been discovered (Wu *et al.*, 2001; Johnson *et al.*, 2011).

ORF5, one of the most divergent regions of the viral genome, encodes glycoprotein 5 (GP5), a glycosylated protein located on the cell surface. GP5 contains decoy epitope (DCE) and primary neutralizing epitope (PNE) and induces pathogenesis including host cell invasion and programmed cell death (Sur *et al.*, 1998). Due to its high divergence, ORF5 plays an important role in genetic variations of PRRSV (Murtaugh *et al.*, 1998) and has been used extensively to study the genetic diversity and evolution of the virus.

PRRSV is classified into 2 genetically distinct groups based on the continent where the virus was first discovered, genotypes 1 (or European) and 2 (or North American). The genomes of the two PRRSV genotypes are markedly different, with genetic similarities of only 60% and 56% at the nucleotide and amino acid levels, respectively (Thuy *et al.*, 2013). Since their emergence, both genotypes have developed independently on each respective continent (Nelsen *et al.*, 1999). However, the co-existence of both genotypes has been increasingly reported in several Asian countries, including China, Korea, Vietnam and Thailand, over the last few years (Thanawongnuwech *et al.*, 2004; Kim *et al.*, 2010; Lee *et al.*, 2010; Chen *et al.*, 2011; Nilubol *et al.*, 2014).

The first PRRSV isolate, belonging to genotype 2, was reported in Thailand in 1996 (Damrongwatanapokin *et al.*, 1996). A subsequent study surveying the genetic diversity of PRRSV in Thailand reported that both genotypes 1 and 2 co-exist in the swine population, with genotype 1 isolates being more dominant (Thanawongnuwech *et al.*, 2004; Tummaruk *et al.*, 2013). However, a study reporting in 2012 demonstrated that both genotypes of PRRSV were concurrently present in the Thai swine population (Nilubol *et al.*, 2012). Nonetheless, both genotypes have been continuously and separately developing, resulting in the development of their own clusters, separate from other Asian PRRSVs (Nilubol *et al.*, 2013). However, the isolate dominance observed was in contrast to the previous report, i.e., genotype 2 was found to be dominant over genotype 1. This observation raises the question of whether genotype 2

viruses will eventually become dominant over genotype 1 viruses in herds in which the two genotypes co-exist, with reduced (or no) outbreaks of novel genotype 1 viruses. Therefore, the objectives of the study were to investigate the dynamics and evolution of genotype 1 PRRSV following its introduction into a herd concurrently infected with both genotypes 1 and 2. In addition, the evolution rate of PRRSV in the herd following the PRRSV outbreak was compared to that prior to the outbreak.

## Materials and Methods

**Herd information:** The study was conducted in a 1,700-sow swine herd, a one-site farrow-to-finish production located in the western region of Thailand. The herd was selected based on the permission of the herd owner. In the studied herd, there were six buildings designated for breeding, gestation and farrowing for the breeding herd. Half of each building was designated for breeding and gestating activities and the other half was designated for farrowing activity. The farrowing facilities operated all-in/all-out on a weekly basis and there was one week of downtime between transitions. 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 meters away.

All sows were artificially inseminated onsite using PRRSV-negative semen. PRRSV-free boar studs were housed at a location five kilometers away from the breeding herd. Semen was tested by polymerase chain reaction (PCR) prior to insemination. Replacement gilts were mainly internally produced and housed with nursery and finishing pigs. These replacements were moved to a gilt developing unit located adjacent to the finishing facilities at 18 weeks of age and introduced to the breeding herd at 32-33 weeks of age.

The PRRSV status of the studied herd was stable/inactive. PRRSV seroconversion, as measured by Idexx ELISA, was evidenced in finishing pigs and both genotypes of PRRSV were detected in the studied herd. PRRSV control was conducted through acclimatization of internal replacement gilts with culled multiparous sows. During the previous three years, the studied herd was a closed herd with no history of external gilt introduction. However, prior to the PRRSV outbreak, grandparent gilts from an external source were imported into the studied herd to improve its genetic program. Soon after their introduction, signs of high fever and loss of appetite were observed in conjunction with abortion and sow mortality which were later observed in the breeding herd. The farrowing rate at the 9<sup>th</sup> month was decreased from 84.0% to 77.4%, and PRRSV was isolated.

**Experimental design:** The study was performed over the course of 19 months. Serum samples were randomly and cross-sectionally collected 10 times (every two months). At each sampling time, five blood samples were collected from each of four population groups: replacement gilts, suckling pigs, nursery pigs

and finishing pigs. Sera were separated and assayed for the presence of viruses by PCR.

**PCR and sequencing:** Total RNA was extracted from serum samples using Nucleospin® RNA Virus (Macherey-Nagel Inc., Duren, Germany) in accordance with the manufacturer's instructions. The extracted RNA was converted to cDNA using M-MuLV Reverse Transcriptase (New England BioLabs Inc., MA, USA). ORF5 was amplified from type 1 and 2 progeny viruses using previously reported primers (Nilubol *et al.*, 2014), and PCR amplification was performed using *Taq* high-fidelity DNA polymerase (Invitrogen™, Carlsbad, CA, USA). The amplified PCR products were purified using a PCR purification kit (Macherey-Nagel, Düren, Germany). Sequencing was performed at Biobasic Inc., (Markham, Ontario, Canada) using an ABI Prism 3730XL DNA sequencer.

**Sequence analysis:** ORF5 sequences were aligned using the CLUSTALW method (Thompson *et al.*, 1994). The phylogenetic trees of the genotype 1 and 2 isolates were constructed separately to investigate the genetic relationship between the progeny viruses. Each phylogenetic tree was constructed based on non-redundant ORF5 sequences of PRRSV isolates collected six months prior to and up to 12 months after the PRRSV outbreak. Moreover, the type 1 prototype virus, Lelystad Virus (LV; accession number M96262) and the genotype 1 MLV (Porcine PRRSV; MSD, Netherlands, accession number AY743931) were included in the analysis of the genotype 1 isolates. The type 2 prototype virus (VR-2332; accession number AY150564) was incorporated into the phylogenetic analysis of the genotype 2 isolates. Neighbor-joining trees with 1,000 bootstrap replicates were also constructed from the aligned nucleotide sequences using MEGA6. Multiple sequence alignments of the amino acid sequences were generated using BioEdit. Pairwise sequence identity percentages were further assessed using the same software.

**Recombination analysis:** Alignments were investigated for recombination events using Recombination Detection Program (Martin *et al.*, 2005). Recombinants were further analyzed using the genetic algorithm for recombination detection (GARD) for potential recombination breakpoints (Kosakovsky Pond *et al.*, 2006) and SimPlot to determine the percent identity of a query sequence against a panel of reference sequences (Lole *et al.*, 1999).

**Evolution analysis:** ORF5 sequences collected seven months prior to the PRRSV genotype 1 outbreak were used as pre-outbreak sequences in the genetic analysis. The remaining sequences were considered post-outbreak sequences. Putative N-glycosylation sites of the deduced amino acid sequences were predicted using the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc>). The nucleotide substitution rate and divergence time of the genotype 1 and 2 isolates during the PRRSV genotype 1 outbreak were calculated by Bayesian Markov chain Monte Carlo (BMCMC) analysis using BEAST v.1.7.4 (Drummond and Rambaut, 2007). The Markov chain

Monte Carlo (MCMC) chain length was adjusted for each dataset and then analyzed with Tracer v.1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>) with a discarded burn-in of 10%. The results were inspected to confirm that stationarity was reached in all cases and the effective sample sizes (ESS) were all greater than 200.

**Statistical analysis:** Data from repeated measurements were analyzed using multivariate analysis of variance (ANOVA). Continuous variables were analyzed by ANOVA to determine whether there were significant differences between treatments. If the p-value in the ANOVA table was < 0.05, differences between treatments were evaluated by pairwise comparisons using least significant differences at the P < 0.05 rejection levels.

## Results

**Sequence analysis:** During the 19 months of collection, a total of 181 complete ORF5 sequences from both PRRSV genotypes was detected in the herd. Of these, 149 (82.32%) sequences belong to genotype 1 and 32 (17.17%) to genotype 2. Identical ORF5 sequences were identified and excluded, resulting in 77 type 1 and 1 type 2 unique sequences for further genetic analysis. These non-redundant ORF5 sequences have been deposited in GenBank under accession numbers KX361347-KX361423 (genotype 1 isolates) and KX361346 (genotype 2 isolates).

The sequences were analyzed and a separate phylogenetic tree was constructed for the sequences of each genotype. The phylogenetic tree of the genotype 1 isolates demonstrated that the genotype 1 virus had evolved into three groups, clusters I, II, and III (Fig. 1). The pair-wise nucleotide and amino acid identity values between the clusters range of 85.6-93.8% and 85.1-92.5%, respectively (Table 1). Cluster I consisted of 100 isolates, including isolates identified both pre- and post-outbreak. The presence of cluster I isolates both pre- and post-outbreak suggests that the cluster I isolates were endemic isolates in the studied herd. Cluster II and III consist of 35 and 14 isolates, respectively. The isolates in clusters II and III emerged following the outbreak.

In contrast, the phylogenetic tree of the genotype 2 isolates consists of only 1 cluster, cluster I, which includes 32 isolates (Fig. 2). The pair-wise nucleotide and amino acid identity values among the isolates in the cluster range of 99.8-100.0% and 99.5-100.0%, respectively. The ORF5 sequences observed following the outbreak were nearly identical to the isolates previously detected in the herd prior to the outbreak, as presented by the distribution of pre- and post-outbreak isolates in the cluster.

**Dynamics of PRRSV evolution in a herd and amino acid analysis:** The dynamic evolution of genotype 1 and 2 isolates is presented in Figure 3. For the genotype 1 isolates, cluster I was persistently identified both pre- and post-outbreak. Interestingly, cluster II emerged within a month following the outbreak. In addition, seven months following the outbreak, cluster III also emerged, of which only fourteen sequences were

detected with the disappearance of cluster II. Sporadic amino acid changes were observed in isolates of both clusters. Interestingly, the emergence of novel clusters was primarily observed in piglets (Table 2).

The clusters in genotype 1 were classified based on the number and position of potential glycosylation sites. All of the three clusters of the type 1 isolates had three conserved glycosylation sites at positions 35, 46 and 53 (Fig. 4). The variation in cluster II was observed

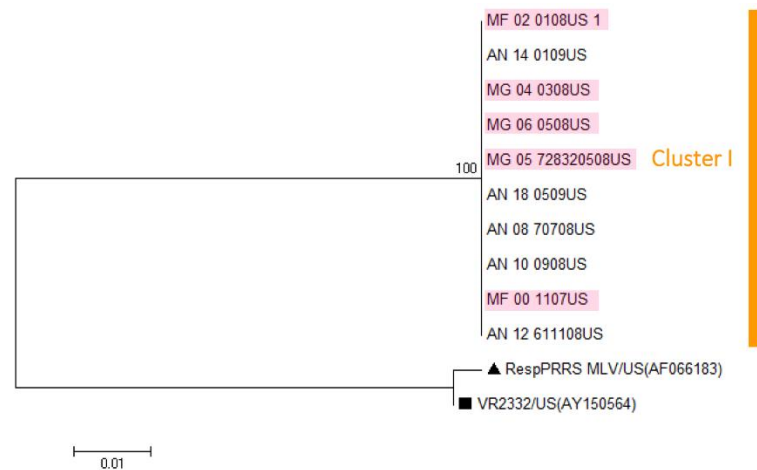
at the position 32 ( $^{32}\text{A}$  to  $^{32}\text{V}$ ), which was located between decoy and neutralizing epitopes. Moreover, the additional N-linked glycosylation positions were also observed in cluster III compared to cluster II, in which the amino acid at position 37 was changed from  $^{37}\text{S}$  to  $^{37}\text{N}$ , following the outbreak (Fig. 4). In contrast, the evolution among the genotype 2 isolates was not observed (Fig. 5), with only cluster I detected both pre- and post-outbreak.



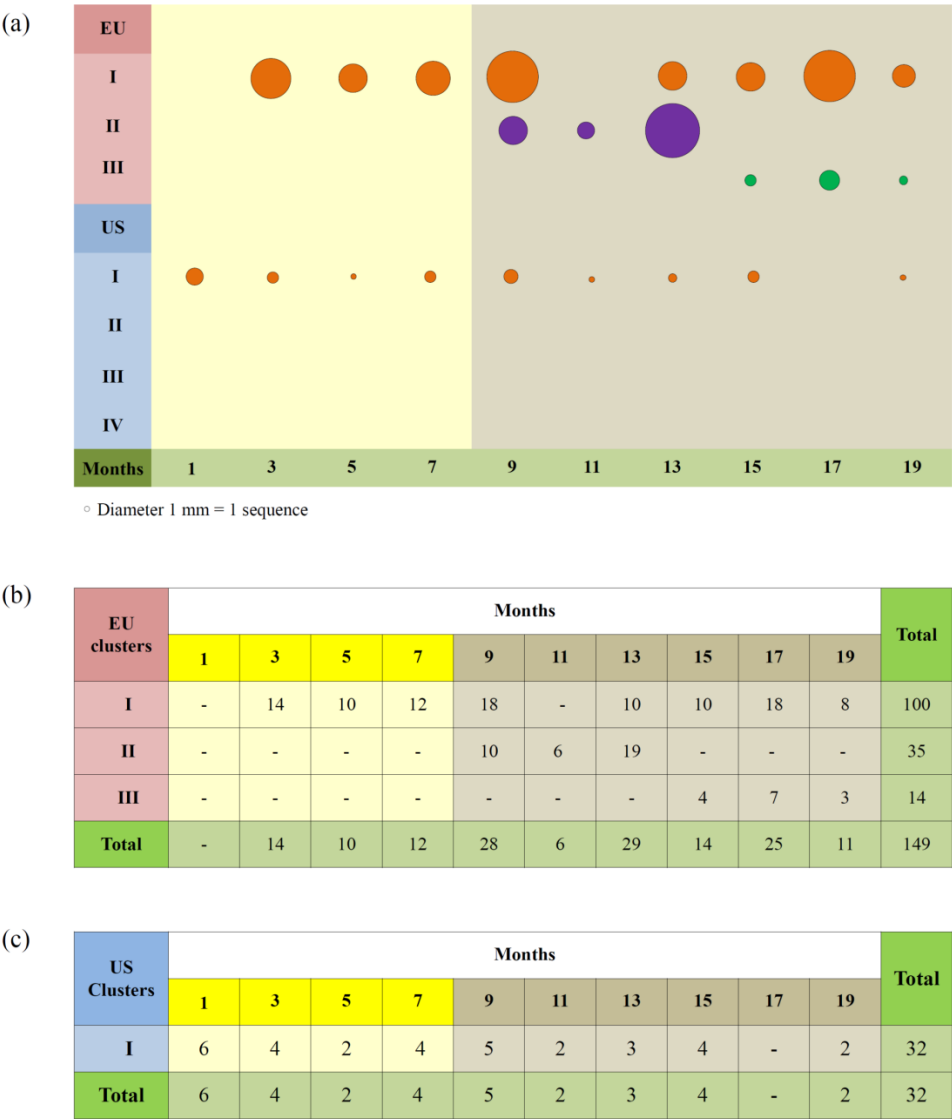
**Figure 1** Neighbor-joining tree of genotype 1 PRRSV ORF5 sequences. The filled square and triangle represent the Lelystad virus and the genotype 1 modified live PRRS vaccine (Porcilis, Netherlands), respectively. ORF5 sequences collected before the PRRSV genotype 1 outbreak are highlighted in pink. The rest of the sequences are from isolates sampled after the outbreak.

**Table 1** The percentage identity of the nucleotide and amino acid sequences of the ORF5 gene between clusters of type 1 isolates

| Nucleotide<br>(Amino acid) | Cluster I                  | Cluster II                 | Cluster III                |
|----------------------------|----------------------------|----------------------------|----------------------------|
| Cluster I                  | 98.3-100.0<br>(95.5-100.0) |                            |                            |
| Cluster II                 | 92.9-93.8<br>(89.6-92.5)   | 99.0-100.0<br>(98.0-100.0) |                            |
| Cluster III                | 85.6-86.9<br>(85.1-88.1)   | 86.1-86.9<br>(89.1-90.0)   | 99.3-100.0<br>(98.5-100.0) |



**Figure 2** Neighbor-joining tree of genotype 2 PRRSV ORF5 sequences. The filled square and triangle represent the genotype 2 prototype virus (VR-2332) and the genotype 2 modified live PRRSV vaccine, respectively. ORF5 sequences collected before the PRRSV genotype 1 outbreak are highlighted in pink. The rest of the sequences are from isolates sampled after the outbreak.

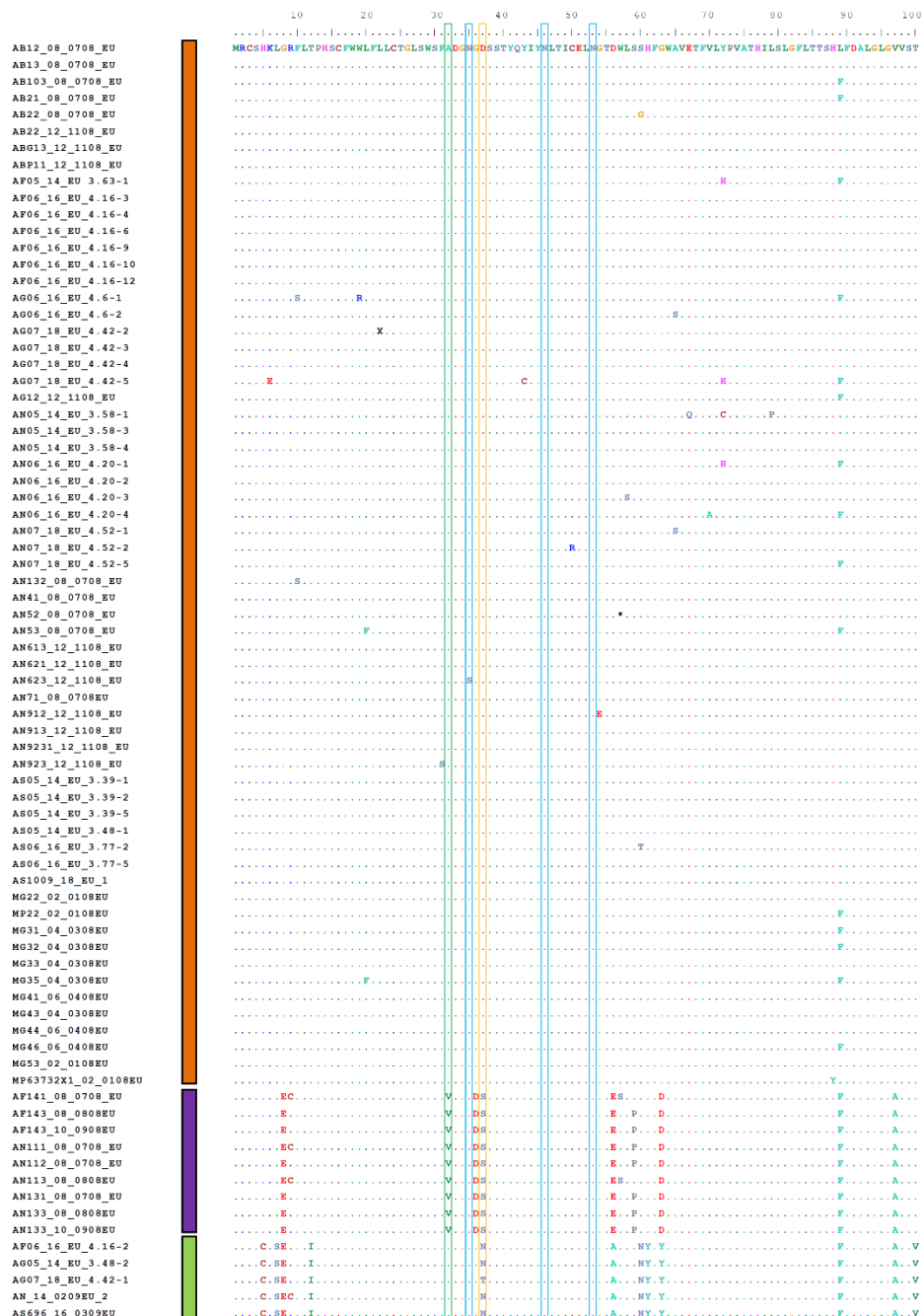


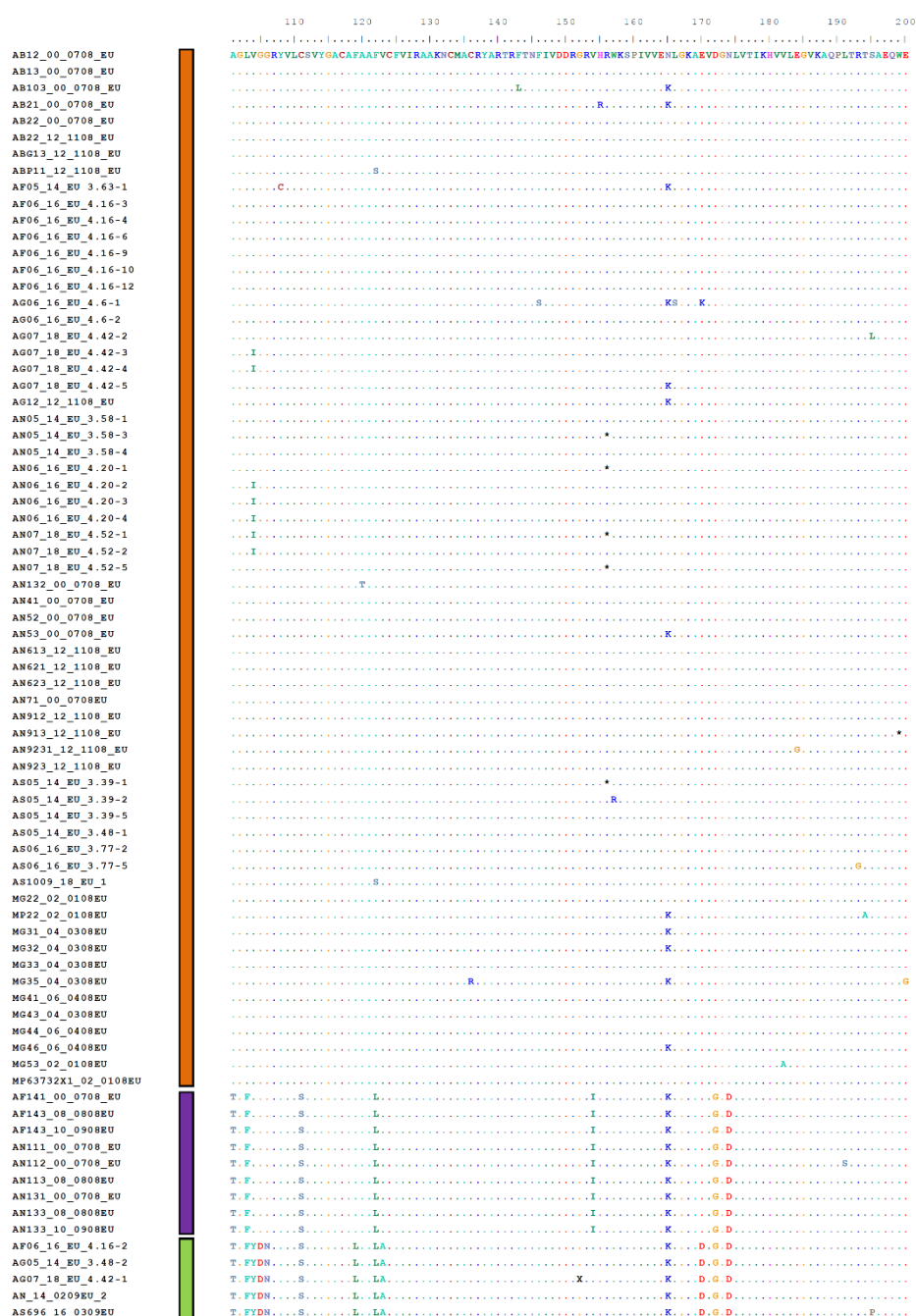
**Figure 3** The distribution of sequence cluster size throughout the sampling period. (a) Sequence clusters at each time point are represented by a circle with the diameter corresponding to the number of isolates. Pre-outbreak collection times are highlighted in yellow and post-outbreak times are highlighted in gray. The cluster assignments are based on the phylogenetic trees displayed in Fig. 1 and 2. The orange, purple and green circles represent clusters I, II and III, respectively. (b) The number of genotype 1 and (c) genotype 2 PRRSV, including those with identical ORF5 sequences, collected at the different sampling times is shown for each sequence cluster.

**Table 2** Pattern of deduced amino acid positions 29-45 primary neutralizing epitope and the number of ORF5 gene of genotype 1 in each cluster by month following the outbreak

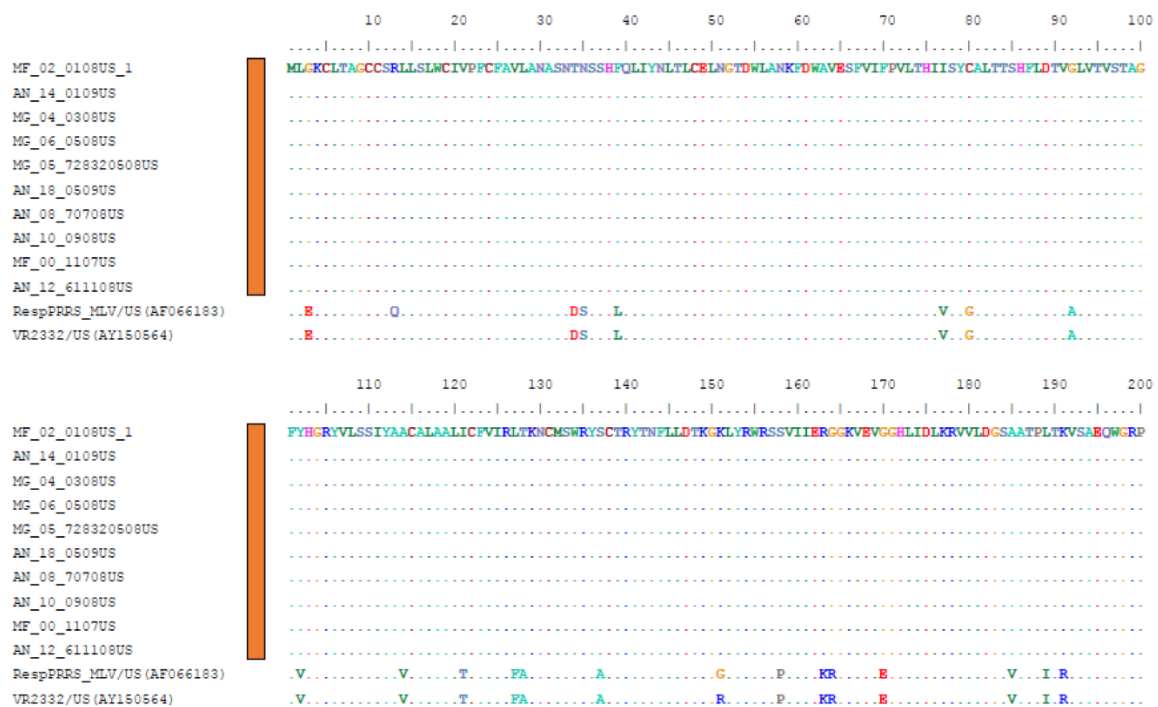
| Cluster | Deduced amino acid positions 29-45 | Months following the outbreak |    |                   |                |                  |                |
|---------|------------------------------------|-------------------------------|----|-------------------|----------------|------------------|----------------|
|         |                                    | 9                             | 11 | 13                | 15             | 17               | 19             |
| I       | WSFADGNGDSSTYQYIY                  | 18                            | -  | 10                | 10             | 18               | 8              |
| II      | WSFVDGNDSSSTYQYIY                  | 10 <sup>a,c</sup>             | 6  | 19 <sup>a,b</sup> | -              | -                | -              |
| III     | WSFADGNGNSSTYQYIY                  | -                             | -  | -                 | 4 <sup>a</sup> | 7 <sup>a,b</sup> | 3 <sup>b</sup> |

Superscripts indicate population groups from which virus was isolated; (a) suckling pigs, (b) nursery pigs and (c) replacement gilts, respectively. No superscript means that virus was isolated from all population groups.

**Figure 4** Multiple alignment of genotype 1 PRRSV ORF5 amino acid sequences. The sequences of the different clusters are labeled with bars of different colors, as follows: orange for cluster I, purple for cluster II and green for cluster III. The blue boxes represent the conserved glycosylation sites among three clusters of the genotype 1 isolate. The green box represents the variation in cluster II. The yellow box represents the additional N-linked glycosylation positions.



**Figure 4 (continue)** Multiple alignment of genotype 1 PRRSV ORF5 amino acid sequences. The sequences of the different clusters are labeled with bars of different colors, as follows: orange for cluster I, purple for cluster II and green for cluster III. The blue boxes represent the conserved glycosylation sites among three clusters of the genotype 1 isolate. The green box represents the variation in cluster II. The yellow box represents the additional N-linked glycosylation positions.



**Figure 5** Multiple alignment of genotype 2 PRRSV ORF5 amino acid sequences. The sequences of cluster I are labeled with an orange bar. The yellow box represents the N-linked glycosylation positions.

**Recombination analysis:** Recombination events, including intra- and inter-genotype, were not evident.

**Evolution analysis:** Evolutionary model parameters, rates of substitution and divergence times were estimated individually for each PRRSV genotype from ORF5 sequences using BMCMC analysis implemented in BEAST. The obtained Bayes factor values suggest that HKY is the optimal substitution model for the genotype 1 and 2 isolates. Thus, the HKY substitution model was tested in the BMCMC analysis with a variety of component parameters. The mean time to the most recent common ancestor (TMRCA), the substitution rate and the 95% highest posterior probability density (HPD) for the different

evolutionary models are shown in Table 3. According to the Bayes factor values, the best fit model for the genotype 1 isolates was the uncorrelated lognormal strict-clock model with a constant-size coalescent tree prior and no rate heterogeneity among sites (Table 3). Under these evolutionary models, the estimated substitution rate for the entire sampling period was  $3.28 \times 10^{-4}$  per site per month for the genotype 1 isolates. For the genotype 2 isolates, the estimated substitution rate for the entire sampling period could not be assessed due to the absence of amino acid changes observed in these isolates. Therefore, the nucleotide substitution rate was only calculated for the genotype 1 isolates.

**Table 3** The estimated TMRCA, the substitution rate and the 95% HPD of ORF5 sequences under different evolutionary models

| Model                   | TMRCA  |         |        | Substitution rate (/site/month) |                       |                       |
|-------------------------|--------|---------|--------|---------------------------------|-----------------------|-----------------------|
|                         | Mean   | 95% HPD |        | Mean                            | 95% HPD               |                       |
| <b>Genotype I virus</b> |        |         |        |                                 |                       |                       |
| HKY + gamma + strict    | 264.24 | 151.00  | 386.59 | $3.28 \times 10^{-4}$           | $2.00 \times 10^{-4}$ | $4.66 \times 10^{-4}$ |
| <b>- Pre-outbreak</b>   |        |         |        |                                 |                       |                       |
| HKY + gamma + strict    | 70.40  | 5.33    | 148.66 | $3.45 \times 10^{-4}$           | $3.77 \times 10^{-7}$ | $8.39 \times 10^{-4}$ |
| <b>- Post-outbreak</b>  |        |         |        |                                 |                       |                       |
| HKY + gamma + strict    | 199.14 | 109.13  | 317.78 | $4.61 \times 10^{-4}$           | $2.44 \times 10^{-4}$ | $6.89 \times 10^{-4}$ |

To determine whether a change in the rate of substitution occurred following the outbreak, BEAST analyses were also conducted separately for the isolates sampled seven months before (months 1, 3, 5, and 7) and after the outbreak (months 9, 11, 13, 15, 17 and 19) using the best evolutionary model chosen in the previous analysis. The rate of nucleotide substitution following the outbreak increased in only genotype 1 with pre- and post-outbreak substitution rates of  $3.45 \times 10^{-4}$  and  $4.61 \times 10^{-4}$  per site per month,

respectively. Moreover, the findings showed significant difference between substitution rates at pre- and post-outbreak ( $P$ -value < 0.0001).

## Discussion

This study investigated the dynamics and evolution of a genetically distinct genotype 1 PRRSV following its introduction into a herd concurrently infected with genotypes 1 and 2. The evolutionary rate

of PRRSV pre- and post-outbreak was calculated and the results of the study demonstrated several interesting points, including no influence of either genotype on the other's development., the introduction of genetically distinct isolates does appear to have influenced the evolution of the viruses of the same genotype, as demonstrated by the emergence of two novel genotype 1 clusters following the outbreak. The evolutionary rate of the virus in the genotype responsible for the outbreak increased after it occurred.

Phylogenetic analysis of the genotype 2 isolates illustrated that only one cluster (cluster I) was detected both pre- and post-outbreak. In contrast, phylogenetic analysis of the genotype 1 isolates demonstrated that they evolved into three groups: clusters I, II and III. Genotype 1 clusters II and III, which are novel clusters, emerged in the herd at one and seven months after the outbreak, suggesting that one of the two novel clusters might potentially be responsible for the outbreak. Moreover, the emergence of cluster II of the genotype 1 virus correlated with a decreased farrowing rate which strengthens the evidence that the cluster II were potentially associated with the outbreak.

The genotype 2 virus was relatively stable. No mutation or substitution was observed prior to/ after outbreak. The results suggested that the introduction of the novel genotype 1 virus had no influence on the development of genotype 2 viruses and the introduction of genotype 1 virus had an influence only on genotype 1 viruses, as characterized by the emergence of novel isolates in the genotype 1 group. This phenomenon is similar, with slight difference, to previous reports in which the introduction of a virus only influences the genetically related genotype and not genetically distinct types. Indeed, a similar pattern has been suggested in which a modified live PRRSV vaccine (MLV) of genotype 2, which is commercially used to control NA PRRSV infection in Thailand, did not influence the development of genotype I isolates following its introduction (Nilubol *et al.*, 2014; Chaikhumwang *et al.*, 2015). Furthermore, the introduction of highly pathogenic PRRSV (HP-PRRSV), characterized by a unique discontinuous deletion of 30 amino acids (AAs) in the Nsp2 region of the genome, to a herd had an influence on the development of genotype 2 isolates by increasing their diversity but not on genotype 1 isolates (Chaikhumwang *et al.*, 2015).

Although the introduction of genotype 1 virus influences only the genetically related genotype, there was some slight difference between the introduction of genotype 1 and 2 virus. The genotype 1 virus did not interfere with the previously existing genotype, unlike the introduction of the genotype 2 virus that interfered with the previously existing genotypes as demonstrated by the increased substitution rate of previously existing isolates (Nilubol *et al.*, 2014) and recombination between the newly introduced and the previously existing isolates (Chaikhumwang *et al.*, 2015).

In the genotype 1 group, the disappearance of cluster II and the emergence of cluster III were observed. We only investigated the amino acid change in the primary neutralizing epitope (PNE). Although amino acid substitution was observed in other regions

(AAs 1-12 and 55-200) of the genotype 1 viruses, their functions remain unknown. The PNE of the genotype 1 virus is located at AAs 37-45, a segment that is similar to that of the NA isolates (Ostrowski *et al.*, 2002; Plagemann *et al.*, 2002; Plagemann, 2004). However, as a recent report had identified a more upstream neutralization epitope, W<sup>29</sup>SFADGN, located in the signal peptide region (AAs 29-35) (Wissink *et al.*, 2003), both segments were analyzed. The first segment, W<sup>29</sup>SFADGN, is conserved among all three clusters of genotype 1 viruses. However, the second segment, located at AAs 37 – 45, shows variation among the three EU clusters, with the D<sup>37</sup> residue being replaced by S<sup>37</sup> in the isolates in clusters II and N<sup>37</sup> in the isolates in clusters III. Considering the fact that cluster II is more closely related to cluster III, though differing in an N-linked glycosylation position, clusters II and III appear to be more closely related to each other than to cluster I. Cluster III developed from cluster II via amino acid changes at an N-linked glycosylation site plays a role in neutralizing immune responses. These results suggest that the cluster I of the genotype 1 virus was potentially responsible for the outbreak and the change into another cluster (cluster III) was potentially to avoid the host immune response. The utilization of an N-linked glycosylation mechanism to avoid the immune system is similar to previous reports regarding genotype 2 viruses (Vu *et al.*, 2011).

An increased rate of evolution was observed only following the outbreak of genotype 1 viruses. The evolution rates of both genotypes in this study was similar prior to the outbreak and the evolutionary rate of the virus was relatively slow in the closed herd system. In contrast, only genotype 1 viruses developed following the outbreak, though this did not occur with the genotype 2 viruses. This result suggests that each PRRSV genotype would evolve more rapidly when an exotic isolate of it genetically related to the genotype was introduced. This supports previous studies concluding that both genotypes evolve at a similar rate and have no influence on the development of the other strain (Nilubol *et al.*, 2013; Nilubol *et al.*, 2014).

One question is how the genotype 1 virus was introduced into the herd. Interestingly, the studied herd had no history of external gilt import during the previous three years and a PRRSV outbreak was detected only recently, after grandparent gilts from an external source were imported into the herd. This pattern suggests that the import of external gilts led to the emergence of cluster II, stressing the importance of strict biosecurity systems in which the introduction of gilts harboring viruses could be harmful to a herd. The housing of gilts in isolation and acclimatization facilities to avoid the shedding period might not be sufficient because PRRSV persistently infects its hosts, which intermittently shed the virus, with transmission to other hosts.

In summary, it was demonstrated in this study that a genetically distinct genotype 1 virus could invade and cause an outbreak in a herd in which genotypes 1 and 2 were concurrently present. The introduction of the novel genotype 1 had no influence on the strain development of another genotype and only influenced its genetically related genotypes, resulting in the emergence of novel isolates in the herd and increasing

the evolutionary rate of the genotype responsible for the outbreak following virus introduction. One of the mechanisms potentially utilized by the genotype 1 virus was a change in the N-linked glycosylation position to avoid recognition by the host immune system.

**Conflict of interest statement:** The authors declare that they have no conflict of interest related to this work.

**Ethical approval:** All applicable international, national and/or institutional guidelines for the care and use of animals were followed

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