Original Article

Genetic evolution of porcine reproductive and respiratory syndrome virus based on ORF5 on 7 Taiwanese pig farms

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus with high genetic variation. Open reading frame 5 (ORF5) is the most suitable genomic region for the identification of strains that have previously circulated on a farm. Herein, 30 ORF5 sequences from 7 Taiwanese pig farms (A, E, F, H, L, S, U) were collected from 2002 to 2014 for analysis of the within-herd genetic evolution of PRRSV analysis. VR-2332, Lelystad, and MD001 (Taiwanese prototype) were selected as reference strains. Nucleotide sequences showing an identity of less than 97% and belonging to different clusters than last isolate from the same farm were considered to represent exotic strains. Our results showed that the identities of these isolates to VR-2332, Lelystad, and MD001 were 84.9-88.4%, 62.1-65.3%, and 85.9-89.6%, respectively. All of these studied strains belonged to PRRSV type II and evolved separately. Two nucleotide sequences from Farm A collected 10 years apart showed 95.5% identity but belonged to the same cluster and were considered to be the same strain endemic to the farm. On the other 6 farms, at least 1 exotic strain was detected during this period. We concluded that in the area, the spread and new invasions of exotic PRRSV strains at the farm level are common events in Taiwan.

Keywords: PRRSV, ORF5, within herd, evolution, Taiwan

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the major swine diseases and causes economic impacts to the industry worldwide (Zimmerman, 2012). The etiological agent of the disease is PRRS virus (PRRSV), which was first identified in the Europe in 1991 and referred to Lelystad virus (Wensvoort *et al*., 1991). Later, in the US, the same virus was isolated and referred to as VR-2332 (Benfield *et al*., 1992). The total cost of productivity losses due to PRRS in the US is estimated to be $664 million annually, including $52.19 per sow, $2.36 per weaned pig, and $4.67 per marketed pig per year (Holtkamp *et al*., 2013).

PRRSV is a positive, single-stranded, enveloped RNA virus belonging to the family *Arteriviridae* in the order *Nidovirales*. Its genome is approximately 15 kb in size and contains at least ten open reading frames (ORFs). ORFs 1a and 1b encode the viral RNA polymerase, and ORFs 2-7 encode structural proteins GP 2-5, M, and N. Two additional structural proteins, E and ORF5a, were recently reported (Wu *et al*., 2001; Johnson *et al*., 2011). According to the genotype, PRRSV can be divided into type I (European, EU) or type II (North American, NA), which share approximately 60% identity over the entire genome (Nelsen *et al*., 1999).

ORF5 encodes the major envelope protein GP5, which is reported to include a neutralizing epitope (Ostrowski et al., 2002). GP5 is located on the surface of the virion and plays an important role in viral infectivity (Dea *et al*., 2000). It contains important immunological domains associated with viral neutralization (Gonin *et al*., 1999). ORF5 is also the most variable gene of PRRSV and has been used to investigate genetic diversity. A phylogenetic tree based on ORF5 showed consistency with the results obtained for the full genome (Kvisgaard *et al*., 2013). Genetic analyses of PRRSV based on ORF5 have been reported in many countries (Thannawongnuwech *et al*., 2004; Cha *et al*., 2006; Thuy *et al*., 2013). Therefore, ORF5 has been widely used to monitor the evolution and molecular epidemiology of PRRSV. ORF5 is the most suitable genome region for the identification of strains that have circulated previously on farms. ORF5 has also been used for diagnostic sequencing for many years in Canada and the US. Nucleotide sequence similarity of 97% or 98% is a useful for indicating relatedness of two virus isolates (Murtaugh, 2012).

The first case of PRRS in Taiwan was recognized in 1991 and reported in 1993 (Chang *et al*., 1993). Two prototypic strains identified in Taiwan, MD001 and WAV, have evolved separately, but the MD001 group is predominant, and invasion by a possible exotic strain suspected to be from the US is thought to have occurred over the last 20 years (Deng *et al*., 2015). Despite the implementation of several commercial vaccines and other tools such as acclimation in Taiwan, the diversity and uncertain within-herd dynamics of PRRSV still make its control difficult in the field. The objective of this study was to investigate the genetic variation of PRRSV based on ORF5 in 7 Taiwanese pig farms from 2002 to 2014 to analyze the disease spread within herds to better understand the dynamics of PRRSV at the farm level.

Materials and Methods

Specimen collection: Clinical samples that were submitted to the Animal Diseases Diagnostic Center (ADDC) of National Pingtung University of Science and Technology (NPUST) and tested positive for PRRSV by real-time polymerase chain reaction described previously (Lin *et al*., 2013) were collected from 2002 to 2014. Seven farms that submitted more than two PRRSV-positive samples included in this dataset were selected for this study. In totally, 29 samples were obtained from these farms.

ORF5 amplification and sequencing: The ORF5 gene was amplified using two reported primers (Iseki *et al*., 2011; Xie *et al*., 2013). The PCR products from these samples were cloned with the yT&A cloning kit (Yeastern Biotech, Taiwan) according to the manufacturer's instructions and submitted to Mission Biotech Company (Taipei, Taiwan) for sequencing. The nucleotide sequences were determined from both orientations by an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis: The nucleotide sequences obtained were compared with VR-2332, Lelystad, and MD001 using Clustal W methods in the MegAlign program (DNASTAR Inc., WI, U.S.A.). A phylogenetic tree was constructed via the maximum likelihood method in MEGA 5.05 as described in a previous study (Kimura, 1980). Samples from different years from the same farm were chosen for within-herd genetic variation analysis. Nucleotide sequences with an identity of less than 97% that belonged to a different cluster than the previous isolate from the same farm were considered to represent an exotic strain (Murtaugh, 2012).

Amino acid sequence analysis: Important sites in GP5 of the PRRSV isolates including the decoy epitope (DCE) located at residues 27-30; the primary neutralizing epitope (PNE) at residues 37-45 (Ostrowski *et al*., 2002); residues 32-34, 38-39, 57-59, which significantly influence the susceptibility of mutant viruses to viral neutralizing antibodies (Kim *et al*., 2013); and residues 13 and 151, which are related to virulence (Allende *et al*., 2000), were selected for comparison with the reference strains.

Results

Phylogenetic analysis: In total, 30 ORF5 nucleotide sequences were obtained from 29 PRRSV-positive pigs in this study (Table 1). The phylogenetic tree is shown in Figure 1. All the isolates evolved separately. The nucleotide sequences obtained from farm A (TW1/A/02 and TW25/A/11), despite being isolated 10 years apart and sharing 95.5% identity, belonged to the same cluster and were considered as the same strain endemic to the farm (Table 2).

Table 1 Farms and ORF5 sequences obtained during 2002-2014

Farm[a]	Size (head)	Type[b]	Sampling period (years)	Denomination (Serial Number/Farm/Year)
A	12000	F-F	10	TW1/A/02, TW25/A/11
E	1000	Breeder	2	TW5/E/05, TW8/E/06, TW9/E/06,
F	8000	F-F	7	TW6/F/06, TW7/F/06, TW11/F/07, TW13/F/07, TW36/F/12, TW37/F/12, TW39/F/12, TW46/F/12
H	5000	F-F	7	TW14/H/07, TW27/H/11, TW28/H11, TW75-1/H/13, TW75-2/H/13
L	7000	F-F	5	TW20/L/09, TW33/L/11, TW35/L/11, TW47/L/12, TW48/L/12, TW52/L/13
S	15000	G-F	3	TW32/S/11, TW70/S/13
U	1500	F-F	3	TW38/U/12, TW77/U/14, TW78/U/14, TW79/U/14

[a]All of these farms have a history of vaccination with a modified live PRRSV vaccine (Ingelvac PRRS MLV, Boehringer Ingelheim Animal Health) during this period.
[b]F-F: Farrow to finish; G-F: Grow to finish

Table 2 Analysis of nucleotide sequence identity of ORF5 between different years in strains obtained from Taiwanese pig farms A, E, F, H, L, S and U

Year-Farm	2011-A	2005-E	2006-E	2006- F	2007-F	2012- F	2012-F	2007-H	2011-H	2013-H	2009-L	2011-L	2012-L	2011- S	2012- U	2014-U
2002-A	95.5															
2006-E		90.4-90.9	99.5													
2006-F				99.2	91.7-96.0	92.0-92.2	89.6-91.0									
2007-F					91.9-98.8	93.0-94.6	89.6-95.7									
2012-F						91.7-94.0	91.9-99.8									
2011-H								94.4-94.5	99.5							
2013-H								89.6-89.9	91.4-92.0	99.7						
2011-L											90.4-91.2	97.8				
2012-L											90.2	90.4-91.0	98.5			
2013-L											90.7	90.7-91.5	98.8-99.0			
2013-S														88.9		~~88.9~~
2014-U															88.2-88.7	88.4-99.5

ORF5 nucleotide sequences analysis: All the nucleotide sequences contained 603 nucleotides. The identities within the studied isolates were 86.9-100% (Table 2); those compared to VR-2332, Lelystad, and MD-001 were 84.9-88.4%, 62.1-65.3%, and 85.9-89.6%, respectively. All isolates included in the study belonged to Type II PRRSV (Figure 1).

ORF5 nucleotide sequences analysis during a 5-7 years sampling period: The 8 nucleotide sequences from Farm F were separated into 3 different clusters: TW6/F/06 together with TW7/F/06, TW11/F/07, and TW13/F/07 (95.9-99.2% identity); TW36/F/12 with TW37/F/12 and TW39/F/12 (99.8-100% identity); TW46/F/12 was alone and shared only 91.9% identity to its previous isolate so was considered to be new invaded strain. The 5 nucleotide sequences from Farm H were separated into two clusters: TW14/H/07 together with TW27/H/11 and TW28/H/11; and TW75-1/H/13 with TW75-2/H/13. TW75-1/H/13 was considered a new invaded strain. The 6 nucleotide sequences from Farm L were separated into 3 different groups: TW20/L/09 alone; TW33/L/11 with TW35/L/11; and TW47/L/12 with TW48/L/12, and TW52/L/13. TW33/L/11 and TW47/L/12 shared 91.2% and 90.4% identity, respectively, with their previous isolates and were considered new invaded strains (Table 2).

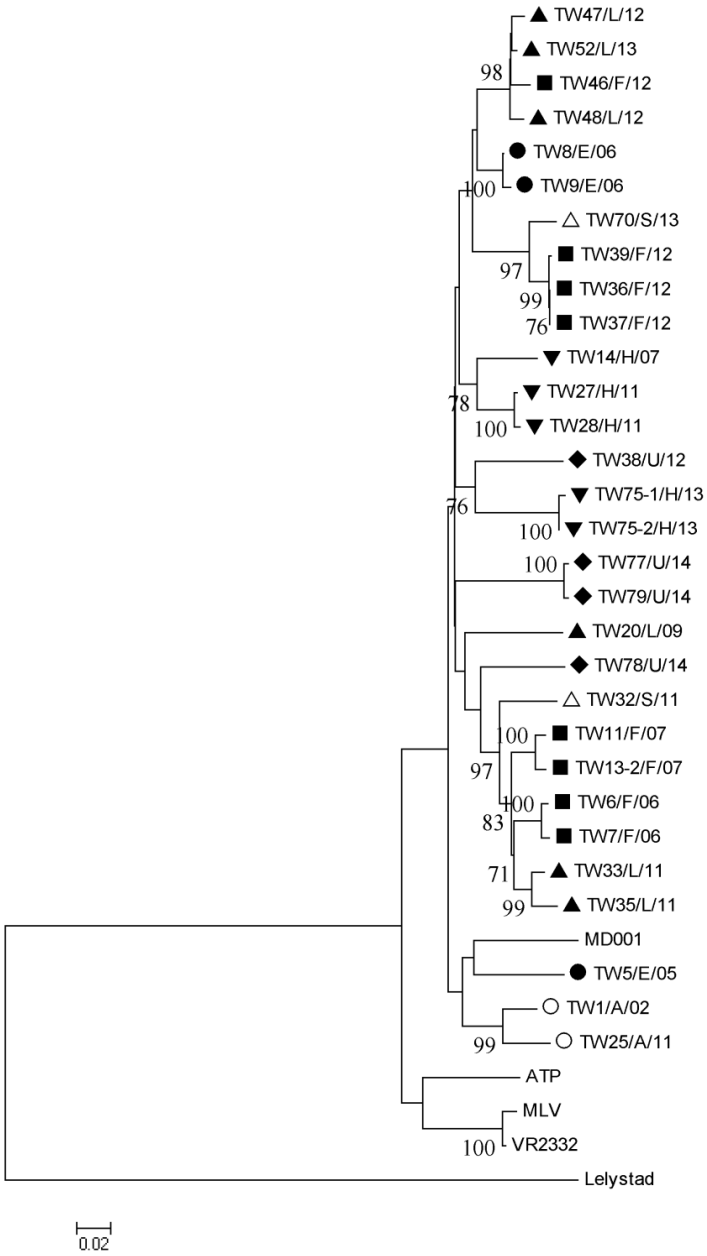

Figure 1 A phylogenetic tree was constructed on the basis of VR-2332, Lelystad, MD001 and 30 Taiwanese PRRSV ORF5 nucleotide sequences. ○, ●, ■, ▼, ▲, △, and ◆ each represent sequences from the same farm.

ORF5 nucleotide sequences analysis during a 2-3 years sampling period: TW8/E/06 was considered an exotic invaded strain compared with the previous isolate TW5/E/05 (90.4% identity) and was considered to be the same strain as TW9/E/06 from Farm E. There was only 88.9% similarity between the 2 nucleotide

sequences from Farm S (TW32/S/11 and TW70/S/13), and they were clustered in different groups, TW70/S/13 could be considered a new invaded strain on the farm. In Farm U: TW38/U/12, TW77/U/14, and TW78/U/14 shared low identity (88.2-88.6%) and clustered into 3 different groups, while TW79/U/14 clustered with TW77/U/14 and shared 99.5% similarity. TW77/U/14 and TW78/U/14 were considered new invaded strains (Table 2).

Amino acid variation in GP5: Amino acid variation in GP5 of these Taiwanese isolates mainly occurs in the signal peptide and ectodomains. Analysis of the amino acid sequences of the invasive and endemic isolates showed that the DCE of most of the isolates was VLVN, except for those of TW25/A/11 is VLVS, TW36/F/12, which were all VFVN, that of TW75-1/H/13, which was VIVN, and that of TW78/U/14, which was ALVN. The primary neutralizing epitope (PNE) was generally SYSQLIYLL, except for those of TW47/L/12 and TW78/U/14, which were SYSQSIYNL and SYFQSIYNL, respectively. Most strains exhibited an R at positions of 13 (except for TW36/F/12, TW/70/S/13, and TW78/U/14, in which this position was occupied by Q) and 151 (except for TW1/A/02, TW25/A/11, and TW70/S/13, in which this position was occupied by K). All of the isolates examined in this study exhibited three N-glycosylation sites at positions 34, 44, 51 (Figure 2).

Discussion

In this study, we collected clinical samples that were submitted to the ADDC of NPUST and were positive for PRRSV from 2002 to 2014. Seven farms from which more than two ORF5 nucleotide sequences obtained in different years during this period were included to analyze the within-herd genetic variation of PRRSV. ORF5 is a highly variable region of the viral genome. It encodes the major envelope protein (GP5) and is widely used for PRRSV diagnostic identification (Kapur *et al*., 1996). In addition, the clustering of the viruses in phylogenetic trees based on the full genome was as expected and resembled the clustering of the isolates in trees were based only on ORF5 (Kvisgaard *et al*., 2013). According to these characteristics, ORF5 is an appropriate target for research on the genetic variation of PRRSV.

All 30 nucleotide sequences examined in this study belonged to Type II PRRSV. This result was similar with those of other studies on PRRSV epidemiology in Taiwan (Shen *et al*., 2010; Wang *et al*., 2008; Deng *et al*., 2015). Some reports from other Asian countries indicate that Type II PRRSV is dominant (Iseki *et al*., 2011; Xie *et al*., 2013; Thuy *et al*., 2013; Faisal *et al*., 2016), while some studies show that both genotypes exist in these countries (Thanawongnuwech *et al*., 2004; King *et al*., 2017). A report from Thailand indicated that two-thirds of Thai isolates were Type I PRRSV and were considered result from the continuous import of swine breeders from both European and North American countries (Thanawongnuwech *et al*., 2004). Several factors may contribute to the domination of Type II PRRSV. First, the use of the commercial modified live vaccine derived from the prototypic American strain VR2332 (Ingelvac PRRS MLV) has been common in Taiwan since its introduction in 1994. Second, no commercial type I PRRSV live vaccine was introduced in Taiwan until 2011. Third, the import of type II PRRSV-infected breeders from abroad plays an important role (Lin *et al*., 2019).

Studies have shown that some field isolates may originate from vaccine viruses (Wang *et al*., 2008; Faisal *et al*., 2016). However, no vaccine-related strains were found in our study. Nilubol *et al*. (2014) indicated that the MLV-related isolates lacked the ability to establish a persistent infection as they disappear within a month. The detection of vaccine derivatives should be an occasional event related to the sampling time and the vaccination protocol of the farm.

Functional domains of GP5 such as its signal peptides, ectodomains, transmembrane regions, and endodomains were identified according to a previous report (Dea *et al*., 2000). Our results regarding the amino acid variation in GP5 from these Taiwanese isolates mainly indicated that such variation occurs in the signal peptide and ectodomains, which is in line with a previous study (Li *et al*., 2009). Kim *et al*. (2013) identified five common variable sites in susceptible and resistant strains and found that changes in amino acid sequences at three sites (32-34, 38-39, and 57-59) located in the N-terminal ectodomain of ORF5 significantly influenced the susceptibility of the mutant viruses to neutralizing antibodies. We found heterogenic at position of 32-34 and 57-59, suggesting that these two sites may be more sensitive as genetic markers.

One of the objectives of this study was to analyze the genetic evolution of PRRSV within farms. The ORF5 nucleotide sequences obtained from Farms A belonged to the same cluster, suggesting that these viruses may be a farm-specific strain and circulate for years. although 97% or 98% nucleotide sequence similarity has been suggested to indicate relatedness of two virus isolates, additional knowledge should be incorporated to avoid blind acceptance of such finding (Murtaugh, 2012). In a PRRSV transmission study carried out by sequentially infecting pigs over a one-year period, the base nucleotide sequence differences varied by 0.7% from the initial inoculum (Chang *et al*., 2002). Field experience and experimental study suggest that PRRSV changes at a rate of approximately 0.5% to 1% per year in the field (Murtaugh, 2012). The two nucleotide sequences from Farm A collected over a period of 10 years showing 95.5% identity may be considered to be the same strain and to be endemic to the farm. Developed their own gilts and less introduction of breeder from outside may be a reason for no exotic strain was detected. When the viruses evolve to cause less virulent but more persistent infections, it may favor survival of the viruses. Residues in GP5 of PRRSV related to virulence have been identified at positions 13 and 151 (Allende *et al*., 2000). As shown in Figure. 2, we found that the 151 residue was K in both amino acid sequences from Farm A, while it was R in the others (except for TW47/L/12, in which it was K). This change may support the persistent infection by the virus.

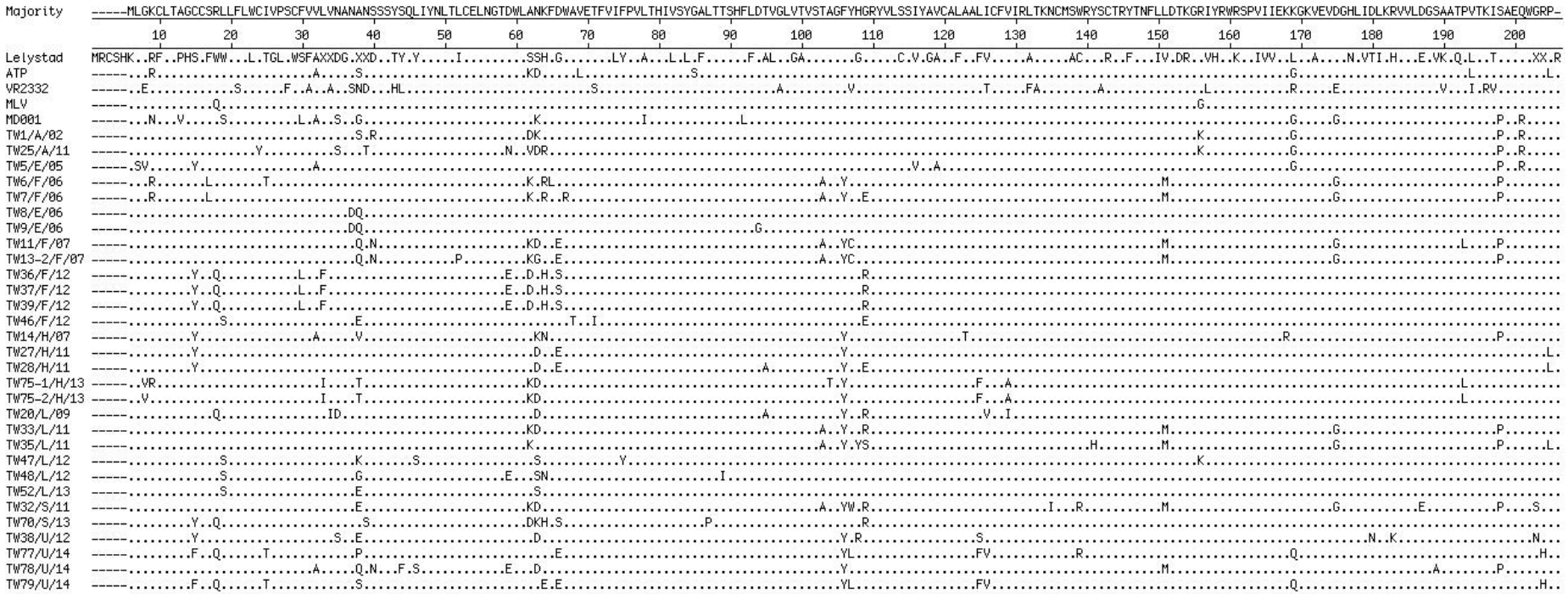

Figure 2 Amino acid sequences of ORF5(GP5) of 30 Taiwanese PRRSV isolates in this study in comparison with VR-2332, Lelystad, and MD001. The signal peptide sequence is underlined. The neutralizing epitope is indicated in a solid line box. Two hypervariable regions are indicated in dotted line boxes.

In contrast to Farm A, the isolates from the other 6 farms showed lower identity and belonged to different clusters, indicating that these farms were invaded by the exotic PRRSV strain. Eight isolates (TW8/E/06, TW36/F/12, TW75-1/H/13, TW33/L/11, TW47/L/12, TW70/S/13, TW77/U/14, TW78/U/14) were considered to be exotic strains on the farm based on two criteria: low identity to the previous isolate from the same farm and clustering into different groups. TW11/F/07 and TW27/H/11 showed 95.9% and 92.5% identity to their preceding isolates TW7/F/06 and TW14/H/07, respectively, which were sampled 1 and 4 years apart. Their genetic similarities were lower than 97%, but they clustered in the same group. These 2 isolates were not considered to be exotic strains in this study since they did not fit both criteria. Further study is necessary to understand the background of their genetic distance. There are several ways for PRRSV to reach a farm, among which the entry of infected animals, particularly gilts and sows, is considered the most common route for virus introduction (Thakur *et al*., 2015). The introduction of breeder pigs from outside the farm without appropriate quarantine is common in Taiwan, which might have contributed to the introduction of new exotic PRRSV strain to these 6 farms. In addition, despite the different sampling period, all the isolates evolved locally based on phylogenetic analysis, suggesting that the invaded viruses were from other areas in Taiwan. Taken together, our results provide evidence supporting the notion that multi-invasion of PRRSV involving other local strains at the farm level is a common event in Taiwan. Farmers should be pay more attention to the external biosecurity concerns such as appropriate quarantine for breeding pigs from domestic pig herds, restricted vehicles (slaughterhouse truck and dead-animal truck) moving around the farm etc.

In conclusion, the 30 isolates examined in this study all belonged to Type II PRRSV and evolved independently. Invasion of an exotic PRRSV strain at the farm level is a common event in Taiwan. This information is provided for swine producers and veterinarians to allow them to adjust their PRRSV control programs and biosecurity protocols.

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