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Full genome sequence analysis of a wild, non-MLV-related type 2 Hungarian PRRSV variant isolated in Europe

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a widespread pathogen of pigs causing significant economic losses to the swine industry. The expanding diversity of PRRSV strains makes the diagnosis, control and eradication of the disease more and more difficult. In the present study, the authors report the full genome sequencing of a Type 2 PRRSV strain isolated from piglet carcasses in Hungary. Next generation sequencing was used to determine the complete genome sequence of the isolate (PRRSV-2/Hungary/102/2012). Recombination analysis performed with the available full-length genome sequences showed no evidence of such event with other known PRRSV. Unique deletions and an insertion were found in the nsp2 region of PRRSV-2/Hungary/102/2012 when it was compared to the highly virulent VR2332 and JXA-1 prototype strains. A majority of amino acid alterations in GP4 and GP5 of the virus were in the known antigenic regions suggesting an important role for immunological pressure in PRRSV-2/Hungary/102/2012 evolution. Phylogenetic analysis revealed that it belongs to lineage 1 or 2 of Type 2 PRRSV. Considering the lack of related PRRSV in Europe, except for a partial sequence from Slovakia, the ancestor of PRRSV-2/Hungary/102/2012 was most probably transported from North-America. It is the first documented type 2 PRRSV isolated in Europe that is not related to the Ingelvac MLV.

Introduction

Porcine reproductive and respiratory syndrome emerged at the same time in Europe (early 1990s, Wenswoort et al., 1991) and North America (late 1980s, Keffaber 1989), and since then, the virus (porcine reproductive and respiratory syndrome virus, PRRSV) has rapidly spread throughout the world, and became endemic in almost every major swine producing country.

PRRSV is a member of the Arteriviridae family within the order Nidovirales (Cavanagh 1997; Faaberg et al., 2011). It has a positive-sense single stranded RNA genome of 15kb in length that encodes 10 open reading frames (Snijder and Meulenberg, 1998; Firth et al. 2011; Johnson et al. 2011). Comparative nucleotide sequence analyses revealed that PRRSV strains can be classified into two distinct genotypes: type 1 (formerly named as European) and type 2 (formerly named as North American). Remarkably, the two genotypes have only 50–60% nucleotide identity (Allende et al. 1999).

A comprehensive phylogenetic study of the North American type 2 PRRSV strains has recently been published that was based on the Bayesian analysis of 8624 ORF5 sequences (Shi et al. 2010b). Based on their results, the authors defined 9 monophyletic lineages within this genotype and established a set of reference sequences representing the principal diversity of type 2 sequences.

Type 2 strains were first introduced to Europe in 1996 by the use of a modified live virus (MLV) vaccine in Denmark (Botner et al, 1997). Soon after its introduction into the population, the MLV strain, a cell culture adapted variant of the type 2 prototype VR2332 strain, spread horizontally and vertically among pigs and herds as well, and showed multiple genetic mutations (Nielsen et al. 2001). The vaccine is currently registered in Germany, Poland, The Netherlands, Belgium, Denmark, Spain and Lithuania, and under special import agreements in Slovakia. According to latest results, confirmed by full genome sequence analyses in Denmark, the vast majority of the type 2 strains found in Europe are genetically related (>95% ORF5 nucleotide identity) to the aforementioned vaccine (Kvisgaard et al., 2013). A more recent study involving numerous type 2 ORF5 sequences from throughout Europe revealed a small group of sequences that are 91–94 % similar to the Ingelvac MLV, and can not unequivocally be attributed to the vaccine (Stadejek et al, 2014).

The aim of our study was to characterize a member of the third group of European type 2 sequences, that are 88% or less similar to the Ingelvac strain on ORF5, confirming the

wild type nature of these strains (Balka et al. 2008). These sequences were first identified in 2005 in multiple sites of a swine breeding company with mild clinical signs of PRRS.

Materials and methods

Origin of the isolate

Lung tissue and lymph node samples were obtained from the carcass of a young growing pig originating from an endemically PRRS positive herd, where our previous investigations verified the presence of type 2 PRRSV (Balka et al., 2008). No signs of an acute outbreak were present. Only mild to moderate respiratory symptoms were observed among the young fatteners. No significant reproductive disorders were reported at the time of sampling.

Cells and viruses

Porcine alveolar macrophages (PAMs) obtained from PRRSV-free piglets were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Sigma-Aldrich, Saint Louis, MO, USA) at 37°C and 5% CO_2. To culture PRRSV-2/Hungary/102/2012, approximately 0.5 g pieces of lung tissue were taken from dead pigs. The lung and tracheobronchial lymph node samples originating from the endemically infected farm were homogenized with Tissue Lyser (Qiagen, Hilden, Germany) in sterile phosphate-buffered saline (PBS) containing antibiotics and antimycotics, to obtain a 50% w/v suspension. After complete homogenization, the samples were centrifuged at 5000×g for 10 min to remove cellular debris. Cell-free supernatants were frozen at –80°C for RNA isolation. PAMs were inoculated with 100 µl of supernatant and incubated for five days. Besides the periodic examination of the cell cultures for the presence of cytopathic effects, real-time RT-PCR analysis (Balka et al., 2009) was also applied on the supernatants to confirm the growth of the virus. Cell-free supernatants were stored at –80°C for RNA isolation.

RNA isolation and cDNA synthesis

RNA was isolated with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from the original tissue homogenates and PAM cell culture supernatants. cDNA was generated using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) kit and a T_{20} primer, according to the protocol of the manufacturer.

Overlapping PCR products for next generation sequencing

The genome of PRRSV-2/Hungary/102/2012 was amplified in five overlapping parts, (similarly to Kvisgaard et al., 2013b), using the Phusion II HotStart PCR kit (Thermo Scientific, Waltham, MA, USA), in 25 µl final volume with 1 µl of cDNA template, in 1× GC buffer in the presence of 4% DMSO. Amplification was performed using the following gradient PCR program: 98°C 1', 35 × [98°C 20", 54-72°C (gradient ramp: 2°C) 20", 72°C 5'], 72°C 5'. The primer pairs used for the amplification of the different fragments are listed in Table 1. Primers were designed using Primer3Plus (Untergasser et al., 2007). PCR fragments were purified from agarose gel slices by the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). For determination of both ends of the genome, the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA) as well as a forward ORF7 primer (Balka et al., 2008) and the T_{20} primer were applied.

Next generation sequencing (NGS)

An equimolar mixture of the overlapping PRRSV PCR products was used as template for next generation sequencing. In brief, a DNA library was prepared using the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Beverly, MA, USA) with the Ion Torrent Xpress barcode adapters (Life Technologies, Carlsbad, CA, USA) according to the protocol recommended by the manufacturers. The emulsion PCR and subsequent template enrichment were carried out with the Ion OneTouch™ Template Kit on a OneTouch v1 instrument and an Ion OneTouch™ ES pipetting robot, respectively. Sequencing was carried out on a 316 chip using the Ion Torrent semiconductor sequencing equipment (Ion Personal Genome Machine® (PGMTM); Life Technologies). Sequences were assembled and aligned with SeqMan Ngen software (Lasergene, Madison, WI, USA).

Phylogenetic analysis

PRRSV whole genome sequences in Genbank, including 16 type 1 and 199 type 2 field, vaccine and laboratory strains, were obtained from the continents of Europe, Asia, and North America (n=215, Table 2). PRRSV whole genome alignment was done with the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004) in Geneious Pro 6.1.7 using default settings. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-266940) is shown. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated

using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 215 genome sequences. All positions containing gaps and missing data were eliminated. There were a total of 12911 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Additional type 2 PRRSV ORF5 sequences were added to the database to enlarge total genetic diversity. ORF5 nucleotide sequences were aligned with MUSCLE and phylogeny was determined by maximum likelihood in MEGA as described above. The closest known relatives to PRRSV-2/Hungary/102/2012 were determined by BLAST analysis in Genbank (Altschul et al., 1990).

Recombination analysis of the complete genome was performed with Recombination Analysis Tools (RAT) (Etherington et al., 2005) using the 215 Genbank accessions as references. Insertion-deletion analysis was visualized by the alignment of the Hungarian isolate to the type 2 prototype strain VR2332 (acc. number: EF536003) and to the highly pathogenic Chinese strain CHN-JXA1 (acc. number: EF112445).

The distribution of N-glycosylation sites was determined using NetNGlyc 1.0 Server web utility (http://www.cbs.dtu.dk/services/NetNGlyc/). The antigenic regions, T and B cell epitopes were determined as described using an amino acid alignment with the reference strains VR2332, Ch-1a (acc. number:AY032626) and NVSL 97-7895 (acc. number: AY545985) (Diaz et al., 2009; de Lima et al., 2006; Mokhtar et al., 2014; Plagemann et al., 2002; Ostrowski et al., 2002; Vashisht et al., 2008; Zhou et al., 2009).

Results

Tissue homogenate supernatants of pig carcasses originating from an endemically infected herd were used to inoculate PAM cells in order to isolate and amplify the PRRSV strain for further analysis. Virus induced cytopathic effect, i.e. cell lysis was observed from the 2nd day post infection and reached 100% by the 5th day. Marked decrease in the C_t values was observed in the real-time RT-PCR reactions performed on the infected cell culture supernatants, compared to the original tissues confirming the increase in viral RNA copy number.

NGS was performed on the equimolar mixture of five overlapping fragments of the entire genome that were amplified by routine RT-PCR applying high fidelity DNA polymerase. After the assembly and alignment of the sequence fragments, the full length

sequence of PRRSV-2/Hungary/102/2012 was found to be 15321 nt in length (GenBank acc. number: KM514315). The untranslated 5' and 3'ends (UTRs) were 189 nt and 151 nt, respectively. The size of the 5'UTR was identical with that of PRRSV DK-2003-2-3 (Genbank Accession No.: KC862584.1) with 12 nt differences in the sequences. The size of the 3'end of PRRSV (PRRSV-2/Hungary/102/2012) was identical with that of PRRSV DK-2004-1-7-Pl (Genbank Accession No.: KC862578.1) with four nt difference.

The phylogenetic analysis of the whole genome of PRRSV-2/Hungary/102/2012 and 215 other full PRRSV genomes showed that it was a novel type 2 PRRSV isolate that was phylogenetically close to the progenitor type 2 PRRSV, and not related to VR2332 or Ingelvac PRRSV MLV (Figure 1.).

The ORF5 dendrogram (Figure 2a) that included a larger dataset of other ORF5 sequences showed that PRRSV-2/Hungary/102/2012 was clustered in lineage 2 PRRSV, which may have originated in Eastern Canada where the earliest isolates were found.

BLAST comparisons of the full genome to Genbank accessions showed that even the most similar strain, VR2385 (JX044140), was only 87% identical. Similarly, BLAST analysis of the ORF5 sequences in Genbank showed that the 11 most similar hits were only 91 to 92% identical, and almost all of these strains were isolates from the early 2000's. Interestingly, the set included a Canadian PRRSV strain IAF 93-2616 (U64932), isolated in 1993, early in the history of PRRSV. To more stringently delineate the origin of PRRSV-2/Hungary/102/2012, the nearest BLAST hits were incorporated with lineage 1 and lineage 2 ORF5 sequences and re-analyzed. The results, shown in Figure 2b, indicate that PRRSV-2/Hungary/102/2012 clusters near the divergence of lineages 1 and 2. The most similar matches to PRRSV-2/Hungary/102/2012, at 92% nucleotide identity (solid circles) were present in both lineages.

To exclude the possibility that PRRSV-2/Hungary/102/2012 was a recombination product involving unknown parental viruses or European progeny of the type 2 PRRSV Ingelvac MLV vaccine, a recombination analysis was performed. The RAT analysis showed no evidence of recombination anywhere in the genomes of all 216 whole genome sequences available to us (data not shown).

PRRSV-2/Hungary/102/2012 has deletions of 10 amino acids (VR3223 aa 314-323) and 9 amino acids (VR2332 aa 792-800) in the nsp2 that were not present in prototype strain VR2332 or the prototype high pathogenicity Chinese strain JXA1 (Figure 3). By contrast, there was a 9 aa insertion in the nsp2 (102HU aa 795-803) that was not present in VR2332 or JXA1. These differences were due to in-frame insertions/deletions at the corresponding coding regions of the genomic RNA (Figure 3).

The antigenic regions (ARs) and glycosylation sites within GP2, GP3, GP4 and GP5 proteins of PRRSV-2/Hungary/102/2012 have been analyzed and compared to references VR2332, Ch-1a and NVSL 97-7895.

At least four B cell epitopes or ARs have been inferred within GP2 (de Lima et al., 2006; Vanhee et al., 2011). The presence of two antigenic regions (AR_{41-55} and $AR_{123-135}$) were confirmed in both type 1 and type 2 PRRSV, while the presence of AR_{92-103}, and the $AR_{206-217}$ were confirmed only in the type 1 prototype strain Lelystad virus (de Lima et al., 2006; Vanhee et al., 2011). However, the two latter regions are conservative between type 1 and type 2 viruses, so it is possible that they are recognized as B cell epitopes in the type 2 PRRSV strains as well. Only one amino acid (aa) substitution was detected in AR_{92-103} between PRRSV-2/Hungary/102/2012 and the reference strains. No aa substitution was detected in $AR_{123-135}$ and $AR_{206-217}$, and four aa substitutions were observed between the less similar NVSL 97-7895 and 102HU in AR_{41-55}. Two N-glycosylation sites are present in GP2 (Das et al., 2010). The position of these glycosylation sites remained similar in all investigated strains though there are some aa changes in the glycosylation recognition sequence of PRRSV-2/Hungary/102/2012 when compared to the reference strains (Figure 4A).

Four experimentally proven antigenic regions were shown within GP3 (de Lima et al., 2006; Zhou et al., 2006, Wang et al., 2014). The AR_{51-105} contains at least five overlapping epitopes (AR_{51-65}, AR_{67-78}, AR_{73-85}, AR_{81-95}, AR_{91-105}) recognized by B cells. Surprisingly, this region is conservative: only five aa differences were detected among the investigated strains and there were only three aa changes between PRRSV-2/Hungary/102/2012 and any of the analyzed strains. AR_{32-46} and $AR_{111-125}$ are completely conserved, with no aa substitutions observed in this region. $AR_{137-159}$, a variable region of GP3, had four aa substitutions in the AR of GP3 of PRRSV-2/Hungary/102/2012. The position of the seven conserved N-glycosylation sites predicted in GP3 is similar to the reference strains (Das et al., 2010) (Figure 4B).

The presence of two T cell epitopes (Díaz et al., 2008) and one B cell epitope (de Lima et al., 2006) were reported within GP4. AR_{51-65} is a hypervariable region of GP4; five aa substitutions were detected in that of PRRSV-2/Hungary/102/2012 compared to the GP4 of the reference strains. A putative T cell epitope$_{7-15}$ (Tce_{7-15}) is recognized by MHCII (described in PRRSV strain L-450) (Díaz et al., 2008). This sequence is very divergent in PRRSV-2/Hungary/102/2012, as it contains three aa substitutions in the 9 aa epitope. $Tce_{170-178}$, recognized by MHCI (same reference as in previous sentence), has two aa substitutions in PRRSV-2/Hungary/102/2012 compared to the other strains. While four N-glycosylation sites

in this protein were recognized in the reference strains (Das et al., 2010), an additional, fifth N-glycosylation site was observed within AR_{51-65} in GP4 of PRRSV-2/Hungary/102/2012 (Figure 4C).

The presence of at least six B cell epitopes (de Lima et al., 2006; Zhou et al., 2009) and three T cell epitopes were reported within GP5. Three antigenic regions in the C-terminus of GP5 protein are conserved (Zhou et al., 2009). $AR_{166-181}$ and $AR_{192-200}$ of PRRSV-2/Hungary/102/2012 have only one aa substitution each and $AR_{149-156}$ is completely conserved compared to the reference strains. Two ARs in the N-terminus of the protein are variable. Three aa substitutions were observed in AR_{1-15} and five aa substitutions in AR_{27-35} (Thaa et al., 2013). A B cell epitope (AR_{37-51}) of the GP5 is highly conserved (Plagemann et al., 2002; Ostrowski et al., 2002), only one aa substitution was found between PRRSV-2/Hungary/102/2012 and Ch1a. The three T cell epitopes are also highly conserved in GP5. Tce_{60-74}, which is recognized by MHCII (described in PRRSV strain L-450), and $Tce_{149-163}$ (described in NADC-9 and NVSL-14) do not contain aa substitutions in PRRSV-2/Hungary/102/2012 when compared to the reference strains (Díaz et al., 2008; Vashisht et al., 2008; Mokhtar et al., 2014). In $Tce_{115-126}$, which is recognized by MHCI (described in PRRSV strain L-450), one aa substitution was observed among the investigated strains (Díaz et al., 2008; Vashisht et al., 2008; Mokhtar et al., 2014). GP5 contains five potential N-glycosylation sites in PRRSV-2/Hungary/102/2012. Two sites (N44 and N51) are highly conserved (Israrul et al., 2006; Meulenberg, 2000) and were found within AR_{37-51}. The other three glycosylation sites (N30, N34 and N35) were within the heterogeneous AR_{27-35} (Figure 4D).

Discussion

Type 2 PRRSV strains are predominant in North America and Asia while in Europe fully sequenced type 2 strains were closely related to Ingelvac PRRS MLV (Kvisgaard et al. 2013a). In case of ORF5 sequences of European type 2 strains they were at least 91% identical to the aforementioned vaccine strain. Our previous results of genetic analysis of a PRRSV Type 2 ORF5 sequence from Hungary and a similar one in Slovakia (one of Hungary's neighboring countries) indicated that there are type 2 strains circulating in these countries that are much more distant to Ingelvac PRRS MLV. In this study we described the genetic, and antigenic characteristics of the complete genome of the first European, wild, type 2 isolate, which is clearly not related to Ingelvac PRRS MLV. Moreover, the genome of PRRSV-

2/Hungary/102/2012 was only 87% identical to the most similar genome available in GenBank proving its unique status.

Phylogenetic analyses performed with the whole genome of PRRSV-2/Hungary/102/2012 and 215 GenBank full genome accessions, as well as ORF5 sequences from type 2 PRRSV isolates globally, revealed that it is a member of an ancient lineage 1 or lineage 2 cluster whose earliest sequence was reported from Eastern Canada in the early 1990's (Brar et al., 2011; Shi et al., 2010b).

Our previous analyses with the use of limited sequences suggested that it might belong to lineage 1 (quite similar to lineage 2) (Stadejek et al., 2014) indicating the importance of using an appropriate, broad-range reference set when genotyping otherwise similar type 2 PRRSV strains.

The Eastern Canadian origin of both lineages 1 and 2 and their cross-border transmission primarily to the North Central USA (Shi et al., 2010b, Brar et al., 2011) suggests that our Hungarian strain may have become established in eastern Europe following the introduction of pigs or germplasm harboring a PRRSV from these regions within the past 10 to 15 years. It then evolved independently for an extended period of time to reach its current level of divergence. As no reliable data are available about pig importation to Hungary from that period, it cannot be excluded that a wild-type 2 PRRSV was introduced elsewhere in the region and then transported to Hungary.

Jackova et al. (2013) published a partial 432 base ORF5 sequence corresponding to the ectodomain of GP5 of a Slovakian isolate from 2003 (strain 36M, acc. number: KC522648), that is 95% similar at nucleotide and 97% similar at amino acid level. As the strain analyzed in our study was isolated from a farm located close to the Slovakian border, and since no other related strains from independent locations were present in public and private databases, this virus is the most likely recent ancestor giving rise to the family of isolates including PRRSV-2/Hungary/102/2012, HU12 (DQ366650) and HU21 (EF406336). The latter two isolates from 2005 showed only 3% nucleotide and 1% amino acid differences in comparison to PRRSV-2/Hungary/102/2012. This relatively slow rate of evolution in one of the most variable part of the PRRSV genome might suggest that use of a type 1 modified live vaccine used in the herd to control endemic type 1 PRRSV did not provoke a strong selective pressure against the type 2 isolate. Hence, it remained conserved over a period of at least 7 years.

Although nsp2 insertions and deletions were present in the genome compared to JAX1 and VR2332, unusual characteristics of pathogenicity or infectivity were not observed under field conditions. However, exact statements on these parameters can only be given after

controlled challenge studies. Whether these deletions and the insertion had a negative effect on tissue tropism, growth rate and/or speed of the genetic change of PRRSV-2/Hungary/102/2012 further investigations are needed.

Antigenic regions of GP2 are relatively conserved, so it is likely that they are functionally important and PRRSV does not tolerate amino acid changes in these regions. The majority of amino acid changes among the investigated strains are positioned in the N-terminal ($GP2_{1-40}$) and the C-terminal ($GP2_{240-256}$) regions of GP2. The N-terminal region contains a hydrophobic stretch of amino acids ($GP2_{27-40}$) that has the potential to be a conditional membrane binding site and/or part of a signal peptide. Although mutations are common in this region, the physicochemical character of the protein remains well conserved, reinforcing the putative functional significance of this site. Similar hypermutability with conserved physicochemical characters can be observed in the amino-terminal hydrophobic regions of all GP proteins ($GP3_{1-30}$, $GP4_{1-20}$ and $GP5_{1-31}$) and all of them are signal peptides (Das et al., 2010; Thaa et al., 2013; Meulenburg, 2000; Kim et al., 2013). The N-glycan addition at N184 in GP2 is critical for recovery of infectious virus but the lack of glycan in N178 does not effect virus growth (Das et al., 2010). A mutation was found within the glycosylation recognition sequence of N184 in PRRSV-2/Hungary/102/2012 compared to the reference strains but this change most probably does not inhibit glycosylation of the site.

Excluding $AR_{137-159}$, the predicted B cell epitopes of GP3 are conserved. The reasons for conservation might be similar to those of GP2. A previous study demonstrated that six sites (Das et al., 2010) of GP3 have glycan moieties from seven potential N-linked glycosylation sites, and N195 is not used for glycosylation. All glycosylation sites are present on GP3 of PRRSV-2/Hungary/102/2012. Mutations are present in two motifs (N29 and N152), but are not likely to affect the glycosylation status.

AR_{51-65}, a hypervariable region in GP4, is considered as neutralization epitope in type 1 PRRSV Lelystad virus. Previous studies have demonstrated that this epitope is susceptible to monoclonal antibody-induced immunoselection *in vitro* (Costers et al., 2010), thus explaining the high variability of this antigenic region. The conserved C terminal anchor ($GP3_{181-197}$) of GP3 overlaps with the variable N-terminus of GP4, which serves as a signal peptide and contain a T-cell epitope (Tce_{7-15}). Interestingly, similar overlaps can be observed between the conserved GP2 C-terminal membrane anchor ($GP2_{210-232}$) and the hypervariable N-terminal signal peptide of GP3. This sequence pattern suggests that the amino acids in membrane anchors of GP2 and GP3 have additional sequence specific functions (e.g. protein-

protein interaction) because the anchor function alone does not necessitate sequential conservation.

All the four potential glycosylation sites of GP4 of VR2332 were shown to be glycosylated. GP4 of PRRSV-2/Hungary/102/2012 contains a fifth N-glycosylation site (N57) in the AR_{51-65}. It can be speculated that this potential glycosylation site might function as an anchor point for glycan shielding.

AR_{1-15} within GP5 is part of signal peptide which is cleaved during peptide processing. AR_{27-35} may function as a decoy epitope, which is hypervariable and is not involved in neutralization. However, recently it has experimentally been proved, that this epitope is only present at very low frequencies as a result of an alternative cleavage site of the signal peptide after the aa 26. (Ostrowski et al., 2002; Thaa et al., 2013). A previous study has demonstrated that aa positions at 32, 33 and 34 of GP5 are under significant positive selection (Delisle et al., 2012). AR_{27-35} contains a functional glycosylation site N34 in NVSL 97-7895, while three potential sites (N30 N34 and N35) can be found in the same region of PRRSV-2/Hungary/102/2012. N30, N34 and N35, together with the highly conserved N44 and N51 compose a very rare combination of N-glycosylation sites on the GP5 of type 2 PRRSVs, less than 1% of the strains contain this pattern (Delisle et al., 2012). The close proximity of N30, N34 and N35 makes it improbable (because of steric inhibition) that all the three sites would be glycosylated together on the same GP5 molecule of PRRSV-2/Hungary/102/2012. On the other hand, considering the positive selection pressure in the region (Delisle et al., 2012), it is also unlikely that the presence of the three glycosylation sites on GP5 would be just a functionless arbitrary event. Most probably these sites are glycosylated in several combinations on different GP5 molecules that compose a set of glycoforms in the PRRSV-2/Hungary/102/2012 envelope similarly as it was shown in the VR2332 virion (Thaa et al., 2013). Alternatively, it cannot be excluded that glycosylation patterns are not under selection, but instead are derivative to changes in ORF5a (Robinson et al., 2013).

As a large majority of the amino acid alterations in GP4 and GP5 of our isolate were found in the previously described antigenic regions, we hypothesize that immunological pressure played an important role in the evolution of the virus. It is possible that an early, fast evolution period might have occurred in the early 2000's when an exponential increase in genetic diversity has been observed among type 2 strains (Shi et al. 2010b). Similar strains were first identified in 2005 by our research team (Balka et al., 2008), and anecdotal information suggested the import of boars from Canada to the herd in previous years. The comparison of these early strains and the recent isolate revealed the slowing down of the

genetic change as only 1% amino acid change occurred in the ORF5 ectodomain in the 7 years between initial sequencing and the recent viral isolation in 2012. These findings indicate a biphasic evolution with a fast, early developmental stage, when the virus may have been surrounded by other type 2 isolates, and a second slower phase after the virus was introduced to this region, and was not influenced by immunity against other type 2 strains.

We conclude that PRRSV-2/Hungary/102/2012 is the first type 2 PRRSV isolated in Europe that belongs to the lineage 1 or 2, and unlike every other European type 2 strain, it is proved to be not related to the Ingelvac MLV. Lineages 1 and 2 were exclusively formed by North American sequences until now. These data suggest that the strain was imported directly from North America during the early stages of PRRSV diversification (most likely from Canada or the North Central USA), and the divergent evolution of the viruses in the two continents resulted in marked genetic differences among PRRSV-2/Hungary/102/2012 and other type 2 viruses.

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References

Allende, R., T. L. Lewis, Z. Lu, D. L. Rock, G. F. Kutish, A. Ali, A.R. Doster, and F. A. Osorio, 1999: North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *J. Gen. Virol.,* 80, 307–315

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, 1990: Basic local alignment search tool. *J. Mol. Biol.,* 215, 403–410.

Ansari I. H., B. Kwon, F, A. Osorio, and A. K. Pattnaik, 2006: Influence of N-Linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J. Virol.* 80, 3994–4004.

Balka G., A. Hornyák, A. Bálint, I. Kiss, S. Kecskeméti, T. Bakonyi, and M. Rusvai, 2008: Genetic diversity of porcine reproductive and respiratory syndrome virus strains circulating in Hungarian swine herds. *Vet. Microbiol.,* 127, 128–135.

Balka G., A, Hornyák, A. Bálint, Z. Benyeda, and M. Rusvai, 2009: Development of a one-step real-time quantitative PCR assay based on primer-probe energy transfer for the detection of porcine reproductive and respiratory syndrome virus. *J. Virol. Methods.,* 158, 41–45.

Bøtner A., B. Strandbygaard K. J. Sørensen, P. Have, K. G. Madsen, E. S. Madsen, and S. Alexandersen, 1997: Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet. Rec.,* 141, 497–499.

Brar, M. S, M. Shi, S. Carman, L. Ge, M. P. Murtaugh and F. C. Leung, 2011: Porcine reproductive and respiratory syndrome virus in Ontario, Canada 1999-2010: diversity and restriction fragment length polymorphism. *J. Gen. Virol.* 92, 1391–1397.

Cavanagh, D., 1997. *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. *Arch. Virol.,* 142, 629–633.

Costers S., D. J. Lefebvre, J. van Doorsselaere, M. Vanhee, P. L. Delputte, H. J. Nauwynck, 2010: GP4 of porcine reproductive and respiratory syndrome virus contains a neutralizing epitope that is susceptible to immunoselection in vitro. *Arch. Virol.,* 155, 371–378.

Das P. B., H. L. Vu, P. X. Dinh, J. L. Cooney, B. Kwon, F. A. Osorio, and A. K. Pattnaik, 2010: Glycosylation of minor envelope glycoproteins of porcine reproductive and respiratory syndrome virus in infectious virus recovery, receptor interaction, and immune response. *Virology*, 410, 385–394.

Delisle B., C. A. Gagnon, M-È. Lambert, and S. D'Allaire, 2012: Porcine reproductive and respiratory syndrome virus diversity of Eastern Canada swine herds in a large sequence

dataset reveals two hypervariable regions under positive selection. *Infect. Genet. Evol.,* 12, 1111–1119.

Díaz I, J. Pujols, L. Ganges, M. Gimeno, L. Darwich, M. Domingo and E. Mateu, 2009: In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. *Virus Res.,* 118, 98–104.

Edgar, R. C., 2004: MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.,* 32, 1792–1797.

Etherington, G. J., J. Dicks, and I. N. Roberts, 2005: Recombination Analysis Tool (RAT): a program for the high-throughput detection of recombination. *Bioinformatics,* 21, 278–281.

Faaberg K. S., U. B. Balasuriya, M. A. Brinton, A.E. Gorbalenya, F. C-C. Leung, H. Nauwynck, E. J. Snijder, T. Stadejek, H. Yang, and D. Yoo, 2012 : Arteriviridae. In: Virus Taxonomy, 9th Report of the International Committee on Taxonomy of Viruses, Elsevier/Academic Press, London.

Firth, A. E., J. C. Zevenhoven-Dobbe, N. M. Wills, Y. Y. Go, U. B. Balasuriya, J. F. Atkins, E. J. Snijder, and C. C. Posthuma, 2011: Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. *J. Gen. Virol.* 92, 1097–1106.

Jacková, A., M. Vlasáková, R. Mandelík, and S. Vilček, 2013: Genetic typing of porcine reproductive and respiratory syndrome virus isolates from central European countries. Acta Virol. 57, 363–368.

Johnson, C. R., T. F. Griggs, J. Gnanandarajah, and M. P. Murtaugh, 2011: Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. *J Gen Virol*. 92, 1107–1116.

Keffaber, K.K. 1989. Reproductive failure of unknown etiology. American Association of Swine Practitioner Newsletter, 1, 1–9.

Kim D-G., C-S. Song, I-S. Choi, S-Y. Park, J-B. Lee, and S-S. Lee, 2013: The signal sequence of type II porcine reproductive and respiratory syndrome virus glycoprotein 3 is sufficient for endoplasmic reticulum retention. *J. Vet. Sci.,* 14, 307–313.

Kvisgaard L. K., C. K. Hjulsager, M. S. Brar, F. C. Leung, and L. E. Larsen, 2013a: Genetic dissection of complete genomes of Type 2 PRRS viruses isolated in Denmark over a period of 15 years. *Vet. Microbiol*., 167, 334–344.

Kvisgaard L. K., C. K. Hjulsager, U. Fahnøe, S. Ø. Breum, Ait-Ali T, and L. E. Larsen, 2013b: A fast and robust method for full genome sequencing of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Type 1 and Type 2. *J. Virol. Methods,* 193, 697–705.

Li J., and M. P. Murtaugh, 2012: Dissociation of porcine reproductive and respiratory syndrome virus neutralization from antibodies specific to major envelope protein surface epitopes. *Virology,* 433, 367–376.

de Lima, M., Pattnaik, A. K., Flores, E. F., and F. A. Osorio, 2006: Serologic marker candidates identified among B-cell linear epitopes of nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus, *Virology*, 353, 410–421.

Meulenberg J. J.M., 2000: PRRSV, the virus. *Vet. Res.,* 31, 11–21.

Mokhtar H., M. Eck, S. B. Morgan, S. E. Essler, J. P. Frossard, N. Ruggli, and S. P. Graham, 2014: Proteome-wide screening of the European porcine reproductive and respiratory syndrome virus reveals a broad range of T cell antigen reactivity. *Vaccine* S0264-410X(14)00592-1. doi: 10.1016/j.vaccine.2014.04.054.

Nielsen H. S., M. B. Oleksiewicz, R. Forsberg, T. Stadejek, A. Bøtner, and T. Storgaard, 2001: Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J. Gen. Virol.,* 82, 1263–1272.

Ostrowski, M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio and O. J. Lopez, 2002: Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J. Virol.* 72, 4241–4250.

Plagemann, P. G. W., R. R. R. Rowland, and K. S. Faaberg, 2002: The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR2332 is located in the middle of the GP5 ectodomain. *Arch. Virol.,* 147, 2327–2347.

Robinson, S.R., J.E. Abrahante, C.R. Johnson, and M.P. Murtaugh, 2013: Purifying selection in porcine reproductive and respiratory syndrome virus ORF5a protein influences variation in envelope glycoprotein 5 glycosylation. *Infect. Genet. Evol.,* 20, 362–368.

Shi M., T. T. Lam, C. C. Hon, R. K. Hui, K. S. Faaberg, T. Wennblom, M. P. Murtaugh, T. Stadejek, and F. C. Leung. 2010a: Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Res.,* 154, 7–17.

Shi, M., T. T. Lam, C. C. Hon, M. P. Murtaugh, P. R. Davies, R. K. Hui, J. Li, L. T. Wong, C. W. Yip, J. W. Jiang, and F. C. Leung, 2010b: Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. *J. Virol.,* 84, 8700–8711.

Stadejek T., G. Balka, Á. Bálint, T. Duinhof, and M. P. Murtaugh, 2014: The Origin and evolution of European PRRSV Type 2 strains. In: Proceedings of the 6th European Symposium of Porcine Health Management Sorrento, Italy, 7–9 May 2014. pp. 87.

Tamura K., and M. Nei, 1993: Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol., 10,* 512–526.

Tamura K., G. Stecher, D. Peterson, A. Filipski, and Kumar S, 2013: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.,* 30, 2725–2729.

Thaa B, B. C. Sinhadri, C. Tielesch, E.Krause, and M. Veit, 2013: Signal peptide cleavage from GP5 of PRRSV: a minor fraction of molecules retains the decoy epitope, a presumed molecular cause for viral persistence. *PLoS One* 8, e65548.

Untergasser A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. Leunissen, 2007: Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.,* 35, W71–74.

Vanhee, M, W. van Breedam, S. Costers, M. Geldhof, Y. Noppe, and H. Nauwynck, 2011: Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use of peptide-specific serum antibodies. *Vaccine,* 29, 4794–4804.

Vashisht K., T. L. Goldberg, R. J. Husmann, W. Schnitzlein, and F. A. Zuckermann, 2008: Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus *Vaccine*, 26, 4747–5753.

Wang X, R. Dang, W. Liu, Z. Yang, E. Du, and S. Zhang, 2014: Antigenic characteristics of glycosylated protein 3 of highly pathogenic porcine reproductive and respiratory syndrome virus. *Virus Res.,* 189, 24–28.

Wensvoort, G., C. Terpstra, J. M. Pol, E. A. ter Laak, M. Bloemraad, E. P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J. M. Broekhuysen, P. L. J. M. Moonen, T. Zetstra, E. A. de Boer, H. J. Tibben, M. F. de Jong, P. de Veld, G. J. R. van`t Groenland, J. A. van Gennip, M. T. Voets, J. H. M. Verheijden, and J. Braamskamp, 1991: Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet. Quart.,* 13, 121–128.

Zhou, Y-J., T-Q. An, Y-X. He, J-X. Liu, H-J. Qiu, Y-F. Wang, and G. Tong, 2006: Antigenic structure analysis of glycosilated protein 3 of porcine reproductive and respiratory syndrome virus. *Virus Res.,* 118, 98–104.

Zhou Y-J., H. Yua, Z-J. Tian, J-X. Liu, T-Q. An, J.-M. Peng, G.-X. Li, Y-F. Jiang, X-H. Cai, Q. Xue, M. Wang, Y.-F. Wang, G.-Z. Tong, 2009: Monoclonal antibodies and conserved antigenic epitopes in the C terminus of GP5 protein of the North American type porcine reproductive and respiratory syndrome virus. *Vet. Mic.,* 138, 1–10.

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Figure legends

Figure 1.

Molecular phylogenetic analysis of PRRSV whole genomes by maximum likelihood method. Lineage designations are from Shi et al., 2010b.

Figure 2.

Maximum likelihood phylogenetic trees for PRRSV-2/Hungary/102/2012 ORF5 (A.) designations are from Shi et al., 2010b. (B.) Representative lineage 1 and 2 sequences including closest Genbank BLAST hits to PRRSV-2/Hungary/102/2012 (arrow). Closed circles are all sequences with 92% sequence similarity. Open circle has 91% similarity. MB CA: Manitoba, Canada; MN, Minnesota; AR, Arkansas; ?, source not provided in Genbank.

Figure 3.

Insertion/deletion analysis of PRRSV-2/Hungary/102/2012 by the alignment to the type 2 prototype strain VR2332 and the Chinese highly pathogenic reference strain JXA-1. (A) Whole genome sequence schematic with open reading frames (B) Expanded view of the nsp2 nucleotide sequence region showing insertions and deletions (C) Amino acid sequence analysis of nsp2 ORF insertion and deletion pattern.

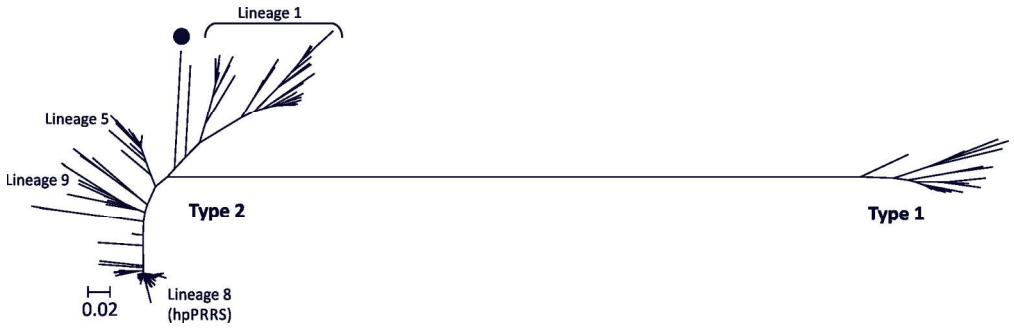
Figure 4.

Alignments and analysis of envelope glycoproteins (GP) 2, 3, 4, and 5 of isolates PRRSV-2/Hungary/102/2012, VR2332, Ch-1a and NVSL 97-7895. Experimentally confirmed and identifiable B-cell epitopes are highlighted by grey boxes. Antigenic regions (AR) are numbered by position. The overlapping regions of ARs are highlighted by dark grey boxes. (A.) GP2. Experimentally confirmed glycolysation sites are underlined and set in bold (de Lima et al., 2006; Vanhee et al.,2011; Das et al., 2010). (B.) GP3. Experimentally confirmed glycosylation sites are underlined and set in bold (de Lima et al., 2006; Das et al., 2010; Zhou et al, 2006; Wang et al., 2014). (C.) GP4. Potential glycosylation sites are underlined and experimentally confirmed sites are set in bold (de Lima et al., 2006; Das et al., 2010;Diaz et al., 2009; Costers et al., 2010). (D.) GP5. Antigenic regions (AR) are numbered by position. T cell epitopes (Tce) are highlighted by open boxes. Potential glycosylation sites are underlined, the experimentally confirmed ones are set in bold (de Lima et al., 2006; Diaz et al., 2009;

Plagemann et al., 2002; Ostrowski et al., 2002; Zhou et al., 2009; Mokhtar et al., 2014; Vashisht et al., 2008).

Table 1. Oligonucleotide primers used for RT-PCR amplification of PRRSV-2/Hungary/102/2012.

Table 2. List of full genome sequences used for the phylogenetic analyses and tree reconstruction.



Molecular phylogenetic analysis of PRRSV whole genomes by maximum likelihood method. Lineage designations are from Shi et al., 2010b.
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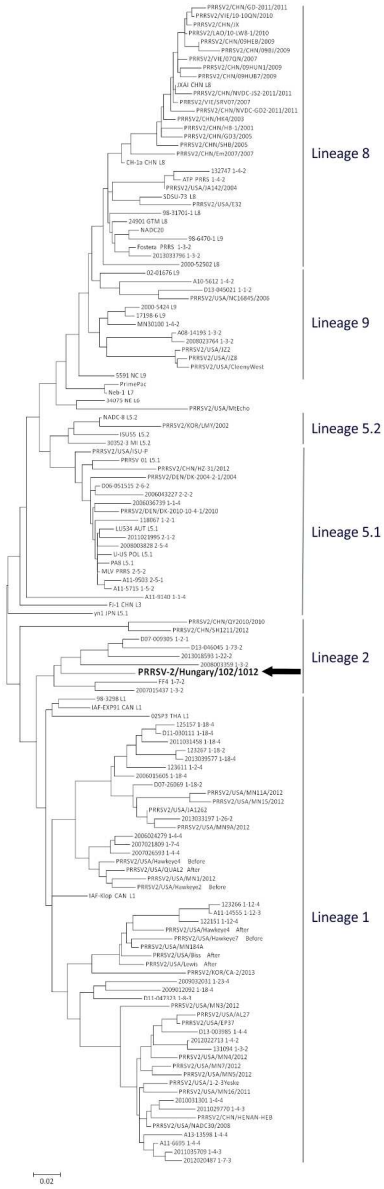

Maximum likelihood phylogenetic trees for PRRSV-2/Hungary/102/2012 ORF5 (A.) designations are from Shi et al., 2010b.
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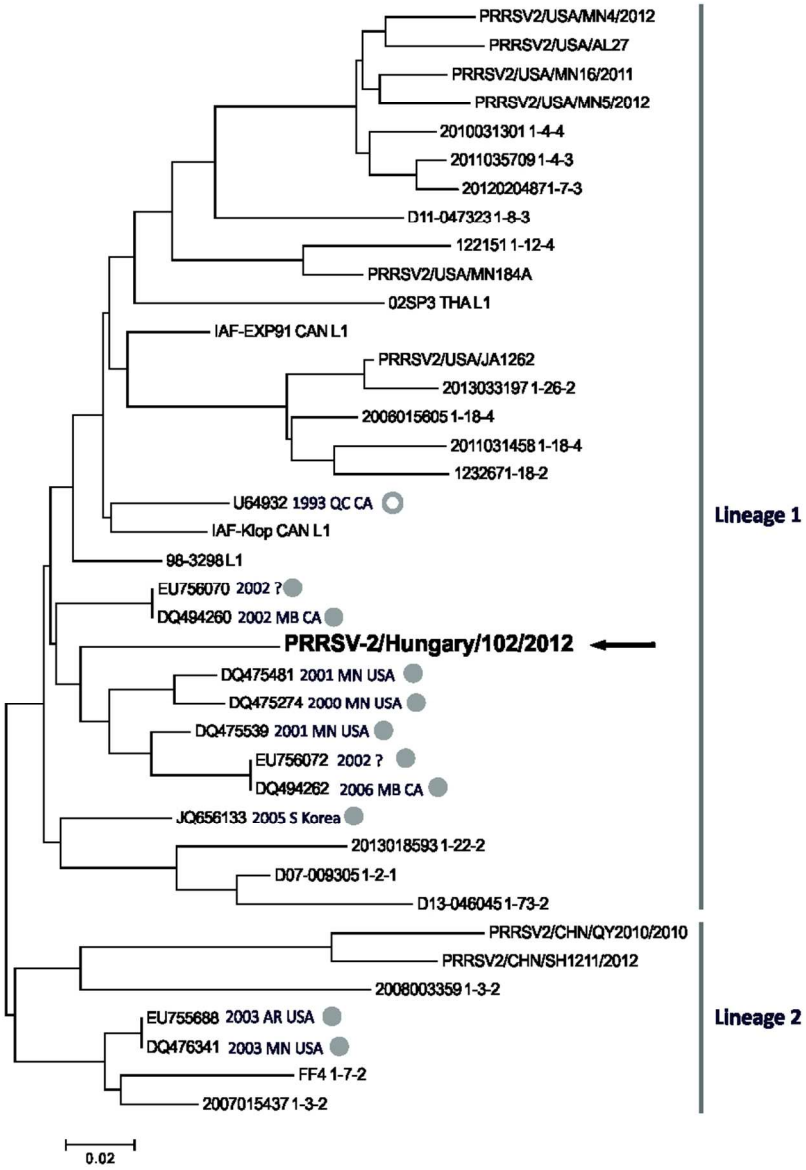
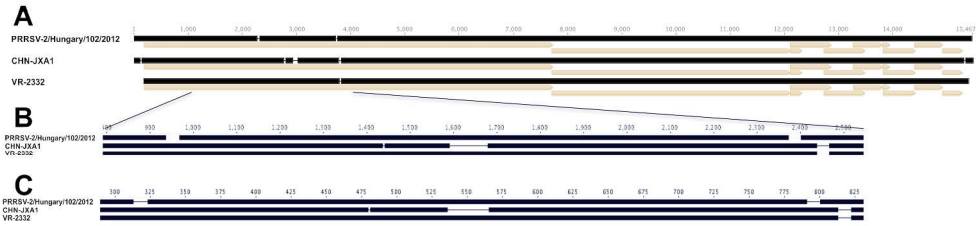
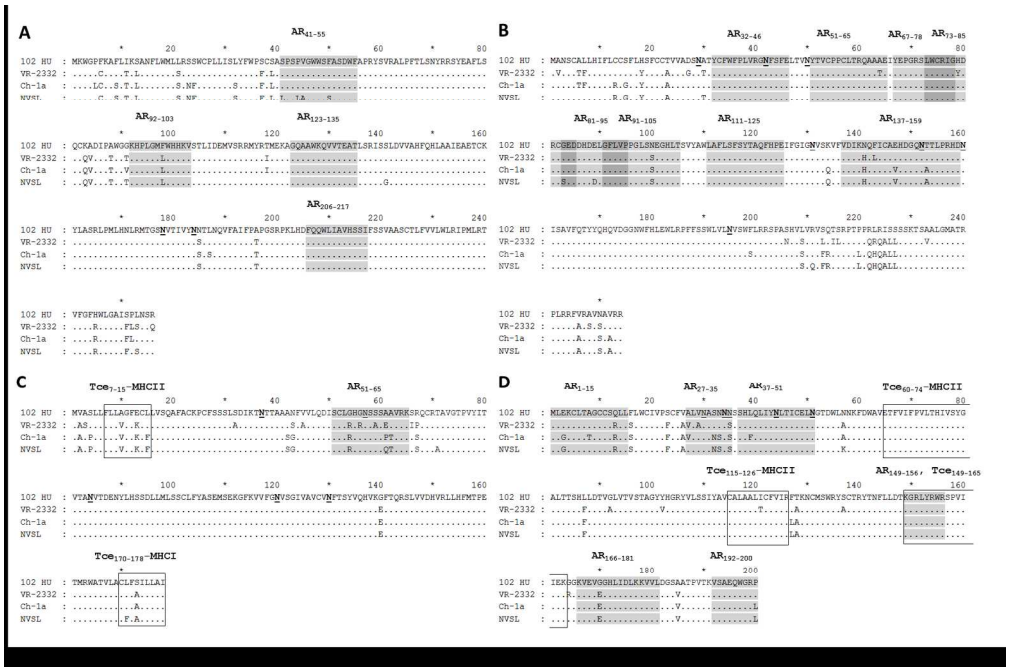


Maximum likelihood phylogenetic trees for PRRSV-2/Hungary/102/2012 ORF5 (B.) Representative lineage 1 and 2 sequences including closest Genbank BLAST hits to PRRSV-2/Hungary/102/2012 (arrow). Closed circles are all sequences with 92% sequence similarity. Open circle has 91% similarity. MB CA: Manitoba, Canada; MN, Minnesota; AR, Arkansas; ?, source not provided in Genbank.
164x229mm (150 x 150 DPI)



Insertion/deletion analysis of PRRSV-2/Hungary/102/2012 by the alignment to the type 2 prototype strain VR2332 and the Chinese highly pathogenic reference strain JXA-1.
(A) Whole genome sequence schematic with open reading frames (B) Expanded view of the nsp2 nucleotide sequence region showing insertions and deletions (C) Amino acid sequence analysis of nsp2 ORF insertion and deletion pattern.

258x61mm (300 x 300 DPI)



Alignments and analysis of envelope glycoproteins (GP) 2, 3, 4, and 5 of isolates PRRSV-2/Hungary/102/2012, VR2332, Ch-1a and NVSL 97-7895. Experimentally confirmed and identifiable B-cell epitopes are highlighted by grey boxes. Antigenic regions (AR) are numbered by position. The overlapping regions of ARs are highlighted by dark grey boxes. (A.) GP2. Experimentally confirmed glycolysation sites are underlined and set in bold (de Lima et al., 2006; Vanhee et al.,2011; Das et al., 2010). (B.) GP3. Experimentally confirmed glycosylation sites are underlined and set in bold (de Lima et al., 2006; Das et al., 2010; Zhou et al, 2006; Wang et al., 2014). (C.) GP4. Potential glycosylation sites are underlined and experimentally confirmed sites are set in bold (de Lima et al., 2006; Das et al., 2010;Diaz et al., 2009; Costers et al., 2010). (D.) GP5. Antigenic regions (AR) are numbered by position. T cell epitopes (Tce) are highlighted by open boxes. Potential glycosylation sites are underlined, the experimentally confirmed ones are set in bold (de Lima et al., 2006; Diaz et al., 2009; Plagemann et al., 2002; Ostrowski et al., 2002; Zhou et al., 2009; Mokhtar et al., 2014; Vashisht et al., 2008).
327x216mm (150 x 150 DPI)

Primer	Sequence	Position
PR_USA_1F	ATGACGTATAGGTGTTGGCTCTATG	1-25
PR_USA_3358R	CAAGCTTAGTCGCATCACATGCCTC	3334-3358
PR_USA_3248F	ACTCAGCTCAAGCCATCATCGACTC	3248-3272
PR_USA_6709R	CAGAGAACACTCCATCGCCAACAAG	6685-6709
PR_USA_6408F	GTCTGCGCAAGTTCTGATGATCAGG	6408-6432
PR_USA_9230R	ATACAGCACGAGGTCGTCCGAATAG	9206-9230
PR_USA_9018F	GTGACTAAGAGAGGTGGCCTGTCGT	9018-9042
PR_USA_12972R	GGAATCCTAGCTCGTCATGATCGTC	12948-12972
PR_USA_12827F	CTTCGAGCTCACGGTGAATTACACG	12827-12851
Pr15_USA_15397R	GGTTCTCGCCAATTAAATCTCACCC	15373-15397

ID	Genebank Accession No.	Strain name	Country
PRRSV2/CHN/NJ-1106/201	JX880029	NJ-1106	CHN
PRRSV2/USA/JA142/2004	AY424271	JA142	USA
PRRSV2/KOR/PL97-1/1997	AY585241	PL97-1	KOR
PRRSV2/USA/NVSL97-7985	AF325691	NVSL 97-7985 IA 1-4-2	USA
PRRSV2/CHN/DC/2010	JF748718	DC	CHN
PRRSV2/CHN/YD/2009	JF748717	YD	CHN
PRRSV2/CHN/SD0901	JN256115	SD0901	CHN
PRRSV2/CHN/SD-CXA/200	GQ359108	SD-CXA/2008	CHN
PRRSV2/CHN/CH-1a/1999	AY032626	CH-1a	CHN
PRRSV2/CHN/BJ-4/2000	AF331831	BJ-4	CHN
PRRSV2/CHN/Henan-A8/2(	KJ534543	Henan-A8	CHN
PRRSV2/CHN/XJu-1/2012	KF815525	XJu-1	CHN
PRRSV2/CHN/HZ-31/2012	KC445138	HZ-31	CHN
PRRSV1/CHN/NVDC-NM1-2	JX187609	NVDC-NM1-2011	CHN
PRRSV2/CHN/YN-2011/201	JX857698	YN-2011	CHN
PRRSV2/CHN/GX1002/201	JQ955658	GX1002	CHN
PRRSV2/CHN/JXM80/2008	GQ499196	JXM80	CHN
PRRSV2/CHN/SDA3/2011	JX878380	SDA3	CHN
PRRSV2/CHN/SCwhn09CD/	JN836553	SCwhn09CD	CHN
PRRSV2/CHN/QYYZ/20011	JQ308798	QYYZ	CHN
PRRSV2/CHN/GM2/2011	JN662424	GM2	CHN
PRRSV2/CHN/SD16/2012	JX087437	SD16	CHN
PRRSV2/CHN/QY2010/201	JQ743666	QY2010	CHN
PRRSV2/CHN/NVDC-JS2-20	JQ715698	NVDC-JS2-2011	CHN
PRRSV2/CHN/NVDC-GD2-2	JQ715697	NVDC-GD2-2011	CHN
PRRSV2/CHN/WUH4/2011	JQ326271	WUH4	CHN
PRRSV2/USA/VR2332/199	PRU87392	ATCC VR-2332	USA
PRRSV1/CHN/NMEU09-1/2	GU047345	NMEU09-1	CHN
PRRSV1/CHN/BJEU06-1/20	GU047344	BJEU06-1	CHN
PRRSV2/CHN/GDQY/2007	GU454850	GDQY2	CHN
PRRSV2/CHN/AH0701/200	GU461292	AH0701	CHN
PRRSV1/CHN/Amervac	GU067771	Amervac	CHN
PRRSV1/NLD/MLV-DV/199	KJ127878	MLV-DV	NLD
PRRSV2/DEN/DK-2011-880	KF183947	DK-2011-88005-A8-Pl	DEN
PRRSV2/DEN/DK-2010-10-	KF183946	DK-2010-10-13-1	DEN
PRRSV2/KOR/CA-2/2013	KF555450	CA-2	KOR
PRRSV1/HUN/9625/2012	KJ415276	9625/2012	HUN
PRRSV2/CHN/HENAN-HEB	KJ143621	HENAN-HEB	CHN
PRRSV1/ESP/Olot-91/1991	KF203132	Olot/91	ESP
PRRSV2/DEN/DK-2004-2-1,	KC862585	DK-2004-2-1	DEN
PRRSV2/DEN/DK-2003-2-3,	KC862584	DK-2003-2-3	DEN
PRRSV2/DEN/DK-2010-10-	KC862583	DK-2010-10-4-1	DEN
PRRSV2/DEN/DK-2008-10-	KC862582	DK-2008-10-1-3	DEN
PRRSV2/DEN/DK-1997-194	KC862576	DK-1997-19407B	DEN
PRRSV2/DEN/DK-2012-01-	KC862575	DK-2012-01-11-3	DEN
PRRSV1/DEN/DK-2003-7-2,	KC862572	DK-2003-7-2	DEN
PRRSV1/DEN/DK-2011-05-	KC862569	DK-2011-05-23-9	DEN
PRRSV1/DEN/DK-2010-10-	KC862568	DK-2010-10-10-3	DEN

PRRSV1/DEN/DK-1992-PRF	KC862566	DK-1992-PRRS-111_92	DEN
PRRSV2/VNM/SRV07/2007	JX512910	SRV07	VNM
PRRSV2/USA/SD95-21/199	KC469618	SD95-21	USA
PRRSV2/CHN/BB0907/200	HQ315835	BB0907	CHN
PRRSV2/CHN/GD-2011/20	KC527830	GD-2011	CHN
PRRSV2/USA/NADC30/200	JN654459	NADC30	USA
PRRSV2/USA/SDSU73	JN654458	SDSU73	USA
PRRSV2/CHN/JX	JX317649	JX	CHN
PRRSV2/CHN/HV	JX317648	HV	CHN
PRRSV2/USA/A2MC2	JQ087873	A2MC2	USA
PRRSV2/CHN/09HUB2/200	JF268683	09HUB2	CHN
PRRSV2/CHN/09HEN1/200	JF268684	09HEN1	CHN
PRRSV2/CHN/09DB2/2009	JF268681	09DB2	CHN
PRRSV2/CHN/09HEB/2009	JF268679	09HEB	CHN
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PRRSV2/CHN/09BJ/2009	JF268676	09BJ	CHN
PRRSV2/CHN/09JS/2009	JF268675	09JS	CHN
PRRSV2/CHN/09HUN1/20(	JF268673	09HUN1	CHN
PRRSV2/CHN/09SC/2009	JF268672	09SC	CHN
PRRSV2/CHN/SX-1/2009	GQ857656	SX-1	CHN
PRRSV2/CHN/SY0909/200	HQ315837	SY0909	CHN
PRRSV2/CHN/NT0801/200	HQ315836	NT0801	CHN
PRRSV2/CHN/BJ0706/2007	GQ351601	BJ0706	CHN
PRRSV1/USA/EuroPRRSV/1	AY366525	EuroPRRSV	USA
PRRSV2/CHN/HN-HW/200(	FJ797690	HN-HW	CHN
PRRSV2/USA/VR2332a	AY150564	VR-2332a	USA
PRRSV2/CHN/HN1	AY457635	HN1	CHN
PRRSV2/USA/MLV	AF159149	MLV RespPRRS/Repro	USA
PRRSV2/CHN/LN1101	KF751238	LN1101	CHN
PRRSV2/CHN/BJ1102/2011	KF751237	BJ1102	CHN
PRRSV2/CHN/SH1211/201	KF678434	SH1211	CHN
PRRSV1/CHN/NVDC-NM3	KC492505	NVDC-NM3	CHN
PRRSV2/CHN/HENAN-XINX	KF611905	HENAN-XINX	CHN
PRRSV2/CHN/HK12/2004	KF287139	HK12	CHN
PRRSV2/CHN/HK4/2003	KF287134	HK4	CHN
PRRSV1/CHN/GZ11-G1/20	KF001144	GZ11-G1	CHN
PRRSV2/CHN/11GZ-GD/20	JX235370	11GZ-GD	CHN
PRRSV2/CHN/10HD-GD/20	JX215553	10HD-GD	CHN
PRRSV2/CHN/JL-0412/201	JX177644	JL-04/12	CHN
PRRSV2/LAO/10-LW8-1/20	JQ663568	10-LW8-1	LAO
PRRSV2/CHN/10-10GX-5/2	JQ663562	10-10GX-5	CHN
PRRSV2/VNM/10-10QN/2(	JQ663556	10-10QN	VNM
PRRSV2/CHN/10-10SD/201	JQ663555	10-10SD	CHN
PRRSV2/CHN/10-10JL/201(	JQ663554	10-10JL	CHN
PRRSV2/CHN/10-10HEB-3/	JQ663553	10-10HEB-3	CHN
PRRSV2/CHN/10-10FUJ-1/2	JQ663546	10-10FUJ-1	CHN
PRRSV2/CHN/10-10BJ-1/2(	JQ663541	10-10BJ-1	CHN
PRRSV2/CHN/10-10JX/201	JQ663540	10-10JX	CHN
PRRSV2/CHN/09HUB7/200	GU168567	09HUB7	CHN

PRRSV2/CHN/GX10-48/20(	JQ309823	GX10-48	CHN
PRRSV2/USA/NC16845/20(	HQ699067	NC16845	USA
PRRSV2/CHN/DY/2007	JN864948	DY	CHN
PRRSV2/CHN/HLJHL/2009	HM189676	HLJHL	CHN
PRRSV2/CHN/GX09-16/20(	HM214913	GX09-16	CHN
PRRSV2/CHN/JN-HS2008	HM016158	JN-HS	CHN
PRRSV2/CHN/ZP-1/2009	HM016159	ZP-1	CHN
PRRSV2/CHN/GDBY1/2008	GQ374442	GDBY1	CHN
PRRSV2/CHN/GDQJ/2007	GQ374441	GDQJ	CHN
PRRSV2/CHN/GD3/2005	GU269541	GD3	CHN
PRRSV2/CHN/SD1-100	GQ914997	SD1-100	CHN
PRRSV2/CHN/KP	GU232735	KP	CHN
PRRSV2/CHN/08SDWF/20(	GU168569	08SDWF	CHN
PRRSV1/THA/01CB1/2001	DQ864705	01CB1	THA
PRRSV2/KOR/PL97-1-LP1/1	AY612613	PL97-1/LP1	KOR
PRRSV2/CHN/Em2007/200	EU262603	Em2007	CHN
PRRSV2/CHN/GS2004/200	EU880443	GS2004	CHN
PRRSV2/CHN/NX06	EU097706	NX06	CHN
PRRSV2/CHN/BJsy06/2006	EU097707	BJsy06	CHN
PRRSV2/CHN/CC-1	EF153486	CC-1	CHN
PRRSV2/USA/Prime Pac/19	DQ779791	Prime Pac	USA
PRRSV2/USA/MN184B	DQ176020	MN184B	USA
PRRSV2/USA/MN184A	DQ176019	MN184A	USA
PRRSV2/CHN/XH-GD	EU624117	XH-GD	CHN
PRRSV2/KOR/LMY/2002	DQ473474	LMY	KOR
PRRSV2/CHN/CH-1R	EU807840	CH-1R	CHN
PRRSV2/USA/Ingelvac ATP	DQ988080	Ingelvac ATP	USA
PRRSV1/KOR/KNU-07/200	FJ349261	KNU-07	KOR
PRRSV2/CHN/SY0608/200(	EU144079	SY0608	CHN
PRRSV2/CHN/WUH3/2008	HM853673	WUH3	CHN
PRRSV2/CHN/08HuN/2008	GU169411	08HuN	CHN
PRRSV2/CHN/HB-1(sh)/20(	AY150312	HB-1(sh)/2002	CHN
PRRSV2/CHN/CWZ-1-F3/2(	FJ889130	CWZ-1-F3	CHN
PRRSV2/CHN/PRRSV03	FJ175689	PRRSV03	CHN
PRRSV2/CHN/GD/2007	EU825724	GD	CHN
PRRSV2/CHN/BJ/2007	EU825723	BJ	CHN
PRRSV2/CHN/HPBEDV	EU236259	HPBEDV	CHN
PRRSV2/CHN/Henan-1	EU200962	Henan-1	CHN
PRRSV2/CHN/Jiangxi-3	EU200961	Jiangxi-3	CHN
PRRSV2/USA/MN/MN184(	EF488739	MN184C	USA
PRRSV2/VNM/07QN/2007	FJ394029	07QN	VNM
PRRSV2/CHN/GD/2006	EU109503	GD	CHN
PRRSV2/CHN/LN/2006	EU109502	LN	CHN
PRRSV2/CHN/SHH/2006	EU106888	SHH	CHN
PRRSV2/CHN/HEB1/2006	EF112447	HEB1	CHN
PRRSV2/CHN/HUN2/2006	EF112446	HUB2	CHN
PRRSV2/USA/MFF_After	EF532819	MFF_After	USA
PRRSV2/USA/Lewis_Before	EF532818	Lewis_Before	USA
PRRSV2/USA/Lewis_After	EF532817	Lewis_After	USA

PRRSV2/USA/ISU-P	EF532816	ISU-P	USA
PRRSV2/USA/Hawkeye7_B	EF532815	Hawkeye7_Before	USA
PRRSV2/USA/Hawkeye7_A	EF532814	Hawkeye7_After	USA
PRRSV2/USA/Hawkeye4_B	EF532813	Hawkeye4_Before	USA
PRRSV2/USA/Hawkeye4_A	EF532812	Hawkeye4_After	USA
PRRSV2/USA/Hawkeye2_B	EF532811	Hawkeye2_Before	USA
PRRSV2/USA/Hawkeye2_A	EF532810	Hawkeye2_After	USA
PRRSV2/USA/FF4_After	EF532809	FF4_After	USA
PRRSV2/USA/FF3_Before	EF532808	FF3_Before	USA
PRRSV2/USA/FF2_After	EF532807	FF2_After	USA
PRRSV2/USA/FF1_Before	EF532806	FF1_Before	USA
PRRSV2/USA/Flagship_Bef	EF532805	Flagship_Before	USA
PRRSV2/USA/Flagship_Aft	EF532804	Flagship_After	USA
PRRSV2/USA/Biss_Before	EF532803	Biss_Before	USA
PRRSV2/USA/Biss_After	EF532802	Biss_After	USA
PRRSV2/CHN/JSyx/2006	EU939312	JSyx	CHN
PRRSV2/CHN/SX2009/200	FJ895329	SX2009	CHN
PRRSV2/CHN/07BJ/2007	FJ393459	07BJ	CHN
PRRSV2/CHN/07HEBTJ/20(	FJ393458	07HEBTJ	CHN
PRRSV2/CHN/07HEN/2007	FJ393457	07HEN	CHN
PRRSV2/CHN/07NM/2007	FJ393456	07NM	CHN
PRRSV2/CHN/TP/2006	EU864233	TP	CHN
PRRSV2/CHN/SHB/2005	EU864232	SHB	CHN
PRRSV2/CHN/CG/2007	EU864231	CG	CHN
PRRSV2/USA/VR2332	EF536003	VR2332	USA
PRRSV2/USA/QUAL2_After	EF536002	QUAL2_After	USA
PRRSV2/USA/QUAL1_Befo	EF536001	QUAL1_Before	USA
PRRSV2/USA/MN30100	EF536000	MN30100	USA
PRRSV2/USA/MFF_Before	EF535999	MFF_Before	USA
PRRSV2/CHN/HUN4	EF635006	HUN4	CHN
PRRSV2/CHN/JXA1/2006	EF112445	JXA1	CHN
PRRSV2/NED/Lelystad viru	M96262	Lelystad virus	NED
PRRSV2/USA/MN9A/2012		MN9A	USA
PRRSV2/USA/MN9B/2012		MN9B	USA
PRRSV2/USA/IA12/2012		IA12	USA
PRRSV2/USA/MN15/2012		MN15	USA
PRRSV2/USA/MN5/2012		MN5	USA
PRRSV2/USA/MN4/2012		MN4	USA
PRRSV2/USA/MN3/2012		MN3	USA
PRRSV2/USA/MN1/2012		MN1	USA
PRRSV2/USA/MN11A/2012		MN11A	USA
PRRSV2/USA/MN6/2012		MN6	USA
PRRSV2/USA/MN14/2012		MN14	USA
PRRSV2/USA/MN2/2012		MN2	USA
PRRSV2/USA/MN11B/2012		MN11B	USA
PRRSV2/USA/MN7/2012		MN7	USA
PRRSV2/USA/IL8/2012		IL8	USA
PRRSV2/USA/MN16/2011		MN16	USA
PRRSV2/USA/MN17A/2012		MN17A	USA

PRRSV2/USA/MN17B/2013	MN17B	USA
PRRSV2/USA/BI- Sample 1	BI- Sample 1	USA
PRRSV2/USA/BI- Sample 2	BI- Sample 2	USA
PRRSV2/USA/JZ1	JZ1	USA
PRRSV2/USA/JZ4	JZ4	USA
PRRSV2/USA/JA1262	JA1262	USA
PRRSV2/USA/AL27	AL27	USA
PRRSV2/USA/EP37	EP37	USA
PRRSV2/USA/1-2-3 Yeske	1-2-3 Yeske	USA
PRRSV2/USA/Cleeny West	Cleeny West	USA
PRRSV2/USA/E32	E32	USA
PRRSV2/USA/JZ2	JZ2	USA
PRRSV2/USA/JZ8	JZ8	USA
PRRSV2/USA/Mt. Echo	Mt. Echo	USA
PRRSV2/USA/K9/2009	K9 (Kingston 2009)	USA
PRRSV2/USA/K10/2010	K10 (Kingston 2010)	USA
PRRSV2/USA/K11/2011	K11 (Kingston 2011)	USA
PRRSV2/USA/K12/2012	K12 (Kingston 2012)	USA
PRRSV2/USA/Bon Homme (Spronk)	Bon Homme (Spronk)	USA
PRRSV2/USA/1784 (IA)	1784 (IA)	USA

Region	Year
Nanjing	2012
	2004
	1997
Iowa	1997
Guangzhou	2010
Guangzhou	2009
	2009
	1999
	2000
Henan	2013
Xinjiang	2012
Hubei	2012
	2011
Yunnan	2011
Guangxi	2010
Shangai	2008
	2011
Sichuan	2009
Guangdong	2011
Guangdong	2011
	2012
Guangdong	2010
Jiangsu	2011
Guangdong	2011
	2011
	1995
Inner Mongolia	2009
Beijing	2006
Guangdong	2007
Anhui	2007
	1999
	2011
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	2013
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	2010

	1992
	2007
South Dakota	1995
Guangxi	2009
	2011
Iowa	2008
	1996
	2010
	2007
	2010
Hubei	2009
Henan	2009
Dongbei	2009
Hebei	2009
Shandong	2009
Beijing	2009
Jiangsu	2009
Hunan	2009
Sichuan	2009
Shanxi	2009
Jiangsu	2009
Jiangsu	2008
Beijing	2007
Iowa	1999
Hunan	2006
Liaoning	2011
Beijing	2011
Shanghai	2012
	2011
Henan	2013
HongKong	2004
HongKong	2003
Guangdong	2011
Guangdong	2011
Guangdong	2010
Jilin	2012
	2010
Guangxi	2010
	2010
Shandong	2010
Jilin	2010
Hebei	2010
Fujian	2010
Beijing	2010
Jiangxi	2010
Hubei	2009

Guangxi	2006
North Carolina	2006
	2007
Heilongjiang	2009
Guangxi	2009
Shandong	2008
Shandong	2009
Guangdong	2008
Guangdong	2007
Guangdong	2005
Guangdong	2008
Chonburi	2001
	1997
	2007
	2007
	2006
Beijing	2006
	1996
Guangdong	2002
	2007
	2006
	2008
Hunan	2008
Hebei	2001
Chongqing	2008
Guangdong	2007
Beijing	2007
Henan	
Jiangxi	
MN	
Quang Nam	2007
Guangdong	2006
Liaoning	2006
Shanghai	2006
Hebei	2006
Hubei	2006

	2005
	2005
	2006
Shanxi	2009
Beijing	2007
Heibei	2007
Henan	2007
Neimeng	2007
Guangdong	2006
Guangdong	2005
Guangdong	2007
Hunan	
Jiangxi	2006
Minnesota	2012
Minnesota	2012
Iowa	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Minnesota	2012
Illinois	2012
Minnesota	2011
Minnesota	2012

Minnesota	2013
Minnesota	2009
Minnesota	2010
Minnesota	2011
Minnesota	2012
Minnesota	
Iowa	