# Transboundary and Emerging Diseases

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

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Manuscripts

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2  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

88  wild type nature of these strains (Balka et al. 2008). These sequences were first identified in

89  2005 in multiple sites of a swine breeding company with mild clinical signs of PRRS.

90

91  **Materials and methods**

92

93  *Origin of the isolate*

94  Lung tissue and lymph node samples were obtained from the carcass of a young growing pig

95  originating from an endemically PRRS positive herd, where our previous investigations

96  verified the presence of type 2 PRRSV (Balka et al., 2008). No signs of an acute outbreak

97  were present. Only mild to moderate respiratory symptoms were observed among the young

98  fatteners. No significant reproductive disorders were reported at the time of sampling.

99

100  *Cells and viruses*

101  Porcine alveolar macrophages (PAMs) obtained from PRRSV-free piglets were cultured in

102  RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin,

103  100 μg/ml streptomycin and 2 mM L-glutamine (Sigma-Aldrich, Saint Louis, MO, USA) at

104  $37°C$ and 5% $CO_2$. To culture PRRSV-2/Hungary/102/2012, approximately 0.5 g pieces of

105  lung tissue were taken from dead pigs. The lung and tracheobronchial lymph node samples

106  originating from the endemically infected farm were homogenized with Tissue Lyser (Qiagen,

107  Hilden, Germany) in sterile phosphate-buffered saline (PBS) containing antibiotics and

108  antimycotics, to obtain a 50% w/v suspension. After complete homogenization, the samples

109  were centrifuged at $5000×g$ for 10 min to remove cellular debris. Cell-free supernatants were

110  frozen at $−80°C$ for RNA isolation. PAMs were inoculated with 100 μl of supernatant and

111  incubated for five days. Besides the periodic examination of the cell cultures for the presence

112  of cytopathic effects, real-time RT-PCR analysis (Balka et al., 2009) was also applied on the

113  supernatants to confirm the growth of the virus. Cell-free supernatants were stored at $−80°C$

114  for RNA isolation.

115

116  *RNA isolation and cDNA synthesis*

117  RNA was isolated with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from the

118  original tissue homogenates and PAM cell culture supernatants. cDNA was generated using

119  Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA)

120  kit and a $T_{20}$ primer, according to the protocol of the manufacturer.

121

122    *Overlapping PCR products for next generation sequencing*

123    The genome of PRRSV-2/Hungary/102/2012 was amplified in five overlapping parts,

124    (similarly to Kvisgaard et al., 2013b), using the Phusion II HotStart PCR kit (Thermo

125    Scientific, Waltham, MA, USA), in 25 μl final volume with 1 μl of cDNA template, in 1× GC

126    buffer in the presence of 4% DMSO. Amplification was performed using the following

127    gradient PCR program: 98°C 1', 35 × [98°C 20", 54-72°C (gradient ramp: 2°C) 20", 72°C 5'],

128    72°C 5'. The primer pairs used for the amplification of the different fragments are listed in

129    Table 1. Primers were designed using Primer3Plus (Untergasser et al., 2007). PCR fragments

130    were purified from agarose gel slices by the QIAquick Gel Extraction Kit (Qiagen, Hilden,

131    Germany). For determination of both ends of the genome, the 5′ RACE System for Rapid

132    Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA) as well as a

133    forward ORF7 primer (Balka et al., 2008) and the $T_{20}$ primer were applied.

134

135    *Next generation sequencing (NGS)*

136    An equimolar mixture of the overlapping PRRSV PCR products was used as template for next

137    generation sequencing. In brief, a DNA library was prepared using the NEBNext® Fast DNA

138    Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Beverly, MA,

139    USA) with the Ion Torrent Xpress barcode adapters (Life Technologies, Carlsbad, CA, USA)

140    according to the protocol recommended by the manufacturers. The emulsion PCR and

141    subsequent template enrichment were carried out with the Ion OneTouch™ Template Kit on a

142    OneTouch v1 instrument and an Ion OneTouch™ ES pipetting robot, respectively.

143    Sequencing was carried out on a 316 chip using the Ion Torrent semiconductor sequencing

144    equipment (Ion Personal Genome Machine® (PGMTM); Life Technologies). Sequences were

145    assembled and aligned with SeqMan Ngen software (Lasergene, Madison, WI, USA).

146

147    *Phylogenetic analysis*

148    PRRSV whole genome sequences in Genbank, including 16 type 1 and 199 type 2 field,

149    vaccine and laboratory strains, were obtained from the continents of Europe, Asia, and North

150    America (n=215, Table 2). PRRSV whole genome alignment was done with the MUltiple

151    Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004) in Geneious

152    Pro 6.1.7 using default settings. The evolutionary history was inferred using the Maximum

153    Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with

154    the highest log likelihood (-266940) is shown. Initial trees for the heuristic search were

155    obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated

156  using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with

157  branch lengths measured in the number of substitutions per site. The analysis involved 215

158  genome sequences. All positions containing gaps and missing data were eliminated. There

159  were a total of 12911 positions in the final dataset. Evolutionary analyses were conducted in

160  MEGA6 (Tamura et al., 2013).

161  Additional type 2 PRRSV ORF5 sequences were added to the database to enlarge total

162  genetic diversity. ORF5 nucleotide sequences were aligned with MUSCLE and phylogeny

163  was determined by maximum likelihood in MEGA as described above. The closest known

164  relatives to PRRSV-2/Hungary/102/2012 were determined by BLAST analysis in Genbank

165  (Altschul et al., 1990).

166  Recombination analysis of the complete genome was performed with Recombination

167  Analysis Tools (RAT) (Etherington et al., 2005) using the 215 Genbank accessions as

168  references. Insertion-deletion analysis was visualized by the alignment of the Hungarian

169  isolate to the type 2 prototype strain VR2332 (acc. number: EF536003) and to the highly

170  pathogenic Chinese strain CHN-JXA1 (acc. number: EF112445).

171  The distribution of N-glycosylation sites was determined using NetNGlyc 1.0 Server

172  web utility (http://www.cbs.dtu.dk/services/NetNGlyc/). The antigenic regions, T and B cell

173  epitopes were determined as described using an amino acid alignment with the reference

174  strains VR2332, Ch-1a (acc. number:AY032626) and NVSL 97-7895 (acc. number:

175  AY545985) (Diaz et al., 2009; de Lima et al., 2006; Mokhtar et al., 2014; Plagemann et al.,

176  2002; Ostrowski et al., 2002; Vashisht et al., 2008; Zhou et al., 2009).

177

178  **Results**

179

180  Tissue homogenate supernatants of pig carcasses originating from an endemically infected

181  herd were used to inoculate PAM cells in order to isolate and amplify the PRRSV strain for

182  further analysis. Virus induced cytopathic effect, i.e. cell lysis was observed from the $2^{nd}$ day

183  post infection and reached 100% by the $5^{th}$ day. Marked decrease in the $C_t$ values was

184  observed in the real-time RT-PCR reactions performed on the infected cell culture

185  supernatants, compared to the original tissues confirming the increase in viral RNA copy

186  number.

187  NGS was performed on the equimolar mixture of five overlapping fragments of the

188  entire genome that were amplified by routine RT-PCR applying high fidelity DNA

189  polymerase. After the assembly and alignment of the sequence fragments, the full length

190   sequence of PRRSV-2/Hungary/102/2012 was found to be 15321 nt in length (GenBank acc.

191   number: KM514315). The untranslated 5' and 3'ends (UTRs) were 189 nt and 151 nt,

192   respectively. The size of the 5'UTR was identical with that of PRRSV DK-2003-2-3

193   (Genbank Accession No.: KC862584.1) with 12 nt differences in the sequences. The size of

194   the 3'end of PRRSV (PRRSV-2/Hungary/102/2012) was identical with that of PRRSV DK-

195   2004-1-7-Pl (Genbank Accession No.: KC862578.1) with four nt difference.

196        The phylogenetic analysis of the whole genome of PRRSV-2/Hungary/102/2012 and

197   215 other full PRRSV genomes showed that it was a novel type 2 PRRSV isolate that was

198   phylogenetically close to the progenitor type 2 PRRSV, and not related to VR2332 or

199   Ingelvac PRRSV MLV (Figure 1.).

200        The ORF5 dendrogram (Figure 2a) that included a larger dataset of other ORF5

201   sequences showed that PRRSV-2/Hungary/102/2012 was clustered in lineage 2 PRRSV,

202   which may have originated in Eastern Canada where the earliest isolates were found.

203        BLAST comparisons of the full genome to Genbank accessions showed that even the

204   most similar strain, VR2385 (JX044140), was only 87% identical. Similarly, BLAST analysis

205   of the ORF5 sequences in Genbank showed that the 11 most similar hits were only 91 to 92%

206   identical, and almost all of these strains were isolates from the early 2000's. Interestingly, the

207   set included a Canadian PRRSV strain IAF 93-2616 (U64932), isolated in 1993, early in the

208   history of PRRSV. To more stringently delineate the origin of PRRSV-2/Hungary/102/2012,

209   the nearest BLAST hits were incorporated with lineage 1 and lineage 2 ORF5 sequences and

210   re-analyzed. The results, shown in Figure 2b, indicate that PRRSV-2/Hungary/102/2012

211   clusters near the divergence of lineages 1 and 2. The most similar matches to PRRSV-

212   2/Hungary/102/2012, at 92% nucleotide identity (solid circles) were present in both lineages.

213        To exclude the possibility that PRRSV-2/Hungary/102/2012 was a recombination

214   product involving unknown parental viruses or European progeny of the type 2 PRRSV

215   Ingelvac MLV vaccine, a recombination analysis was performed. The RAT analysis showed

216   no evidence of recombination anywhere in the genomes of all 216 whole genome sequences

217   available to us (data not shown).

218        PRRSV-2/Hungary/102/2012 has deletions of 10 amino acids (VR3223 aa 314-323)

219   and 9 amino acids (VR2332 aa 792-800) in the nsp2 that were not present in prototype strain

220   VR2332 or the prototype high pathogenicity Chinese strain JXA1 (Figure 3). By contrast,

221   there was a 9 aa insertion in the nsp2 (102HU aa 795-803) that was not present in VR2332 or

222   JXA1. These differences were due to in-frame insertions/deletions at the corresponding

223   coding regions of the genomic RNA (Figure 3).

224     The antigenic regions (ARs) and glycosylation sites within GP2, GP3, GP4 and GP5

225     proteins of PRRSV-2/Hungary/102/2012 have been analyzed and compared to references

226     VR2332, Ch-1a and NVSL 97-7895.

227     At least four B cell epitopes or ARs have been inferred within GP2 (de Lima et al.,

228     2006; Vanhee et al., 2011). The presence of two antigenic regions ($AR_{41-55}$ and $AR_{123-135}$)

229     were confirmed in both type 1 and type 2 PRRSV, while the presence of $AR_{92-103}$, and the

230     $AR_{206-217}$ were confirmed only in the type 1 prototype strain Lelystad virus (de Lima et al.,

231     2006; Vanhee et al., 2011). However, the two latter regions are conservative between type 1

232     and type 2 viruses, so it is possible that they are recognized as B cell epitopes in the type 2

233     PRRSV strains as well. Only one amino acid (aa) substitution was detected in $AR_{92-103}$

234     between PRRSV-2/Hungary/102/2012 and the reference strains. No aa substitution was

235     detected in $AR_{123-135}$ and $AR_{206-217}$, and four aa substitutions were observed between the less

236     similar NVSL 97-7895 and 102HU in $AR_{41-55}$. Two N-glycosylation sites are present in GP2

237     (Das et al., 2010). The position of these glycosylation sites remained similar in all

238     investigated strains though there are some aa changes in the glycosylation recognition

239     sequence of PRRSV-2/Hungary/102/2012 when compared to the reference strains (Figure 4A).

240     Four experimentally proven antigenic regions were shown within GP3 (de Lima et al.,

241     2006; Zhou et al., 2006, Wang et al., 2014). The $AR_{51-105}$ contains at least five overlapping

242     epitopes ($AR_{51-65}$, $AR_{67-78}$, $AR_{73-85}$, $AR_{81-95}$, $AR_{91-105}$) recognized by B cells. Surprisingly, this

243     region is conservative: only five aa differences were detected among the investigated strains

244     and there were only three aa changes between PRRSV-2/Hungary/102/2012 and any of the

245     analyzed strains. $AR_{32-46}$ and $AR_{111-125}$ are completely conserved, with no aa substitutions

246     observed in this region. $AR_{137-159}$, a variable region of GP3, had four aa substitutions in the

247     AR of GP3 of PRRSV-2/Hungary/102/2012. The position of the seven conserved N-

248     glycosylation sites predicted in GP3 is similar to the reference strains (Das et al., 2010)

249     (Figure 4B).

250     The presence of two T cell epitopes (Díaz et al., 2008) and one B cell epitope (de Lima

251     et al., 2006) were reported within GP4. $AR_{51-65}$ is a hypervariable region of GP4; five aa

252     substitutions were detected in that of PRRSV-2/Hungary/102/2012 compared to the GP4 of

253     the reference strains. A putative T cell epitope$_{7-15}$ ($Tce_{7-15}$) is recognized by MHCII (described

254     in PRRSV strain L-450) (Díaz et al., 2008). This sequence is very divergent in PRRSV-

255     2/Hungary/102/2012, as it contains three aa substitutions in the 9 aa epitope. $Tce_{170-178}$,

256     recognized by MHCI (same reference as in previous sentence), has two aa substitutions in

257     PRRSV-2/Hungary/102/2012 compared to the other strains. While four N-glycosylation sites

258    in this protein were recognized in the reference strains (Das et al., 2010), an additional, fifth

259    N-glycosylation site was observed within $AR_{51-65}$ in GP4 of PRRSV-2/Hungary/102/2012

260    (Figure 4C).

261        The presence of at least six B cell epitopes (de Lima et al., 2006; Zhou et al., 2009)

262    and three T cell epitopes were reported within GP5. Three antigenic regions in the C-terminus

263    of GP5 protein are conserved (Zhou et al., 2009). $AR_{166-181}$ and $AR_{192-200}$ of PRRSV-

264    2/Hungary/102/2012 have only one aa substitution each and $AR_{149-156}$ is completely conserved

265    compared to the reference strains. Two ARs in the N-terminus of the protein are variable.

266    Three aa substitutions were observed in $AR_{1-15}$ and five aa substitutions in $AR_{27-35}$ (Thaa et al.,

267    2013). A B cell epitope ($AR_{37-51}$) of the GP5 is highly conserved (Plagemann et al., 2002;

268    Ostrowski et al., 2002), only one aa substitution was found between PRRSV-

269    2/Hungary/102/2012 and Ch1a. The three T cell epitopes are also highly conserved in GP5.

270    $Tce_{60-74}$, which is recognized by MHCII (described in PRRSV strain L-450), and $Tce_{149-163}$

271    (described in NADC-9 and NVSL-14) do not contain aa substitutions in PRRSV-

272    2/Hungary/102/2012 when compared to the reference strains (Díaz et al., 2008; Vashisht et al.,

273    2008; Mokhtar et al., 2014). In $Tce_{115-126}$, which is recognized by MHCI (described in

274    PRRSV strain L-450), one aa substitution was observed among the investigated strains (Díaz

275    et al., 2008; Vashisht et al., 2008; Mokhtar et al., 2014). GP5 contains five potential N-

276    glycosylation sites in PRRSV-2/Hungary/102/2012. Two sites (N44 and N51) are highly

277    conserved (Israrul et al., 2006; Meulenberg, 2000) and were found within $AR_{37-51}$. The other

278    three glycosylation sites (N30, N34 and N35) were within the heterogeneous $AR_{27-35}$ (Figure

279    4D).

280

281    **Discussion**

282

283    Type 2 PRRSV strains are predominant in North America and Asia while in Europe fully

284    sequenced type 2 strains were closely related to Ingelvac PRRS MLV (Kvisgaard et al. 2013a).

285    In case of ORF5 sequences of European type 2 strains they were at least 91% identical to the

286    aforementioned vaccine strain. Our previous results of genetic analysis of a PRRSV Type 2

287    ORF5 sequence from Hungary and a similar one in Slovakia (one of Hungary's neighboring

288    countries) indicated that there are type 2 strains circulating in these countries that are much

289    more distant to Ingelvac PRRS MLV. In this study we described the genetic, and antigenic

290    characteristics of the complete genome of the first European, wild, type 2 isolate, which is

291    clearly not related to Ingelvac PRRS MLV. Moreover, the genome of PRRSV-

292  2/Hungary/102/2012 was only 87% identical to the most similar genome available in

293  GenBank proving its unique status.

294  Phylogenetic analyses performed with the whole genome of PRRSV-2/Hungary/102/2012 and

295  215 GenBank full genome accessions, as well as ORF5 sequences from type 2 PRRSV

296  isolates globally, revealed that it is a member of an ancient lineage 1 or lineage 2 cluster

297  whose earliest sequence was reported from Eastern Canada in the early 1990's (Brar et al.,

298  2011; Shi et al., 2010b).

299      Our previous analyses with the use of limited sequences suggested that it might belong

300  to lineage 1 (quite similar to lineage 2) (Stadejek et al., 2014) indicating the importance of

301  using an appropriate, broad-range reference set when genotyping otherwise similar type 2

302  PRRSV strains.

303      The Eastern Canadian origin of both lineages 1 and 2 and their cross-border

304  transmission primarily to the North Central USA (Shi et al., 2010b, Brar et al., 2011) suggests

305  that our Hungarian strain may have become established in eastern Europe following the

306  introduction of pigs or germplasm harboring a PRRSV from these regions within the past 10

307  to 15 years. It then evolved independently for an extended period of time to reach its current

308  level of divergence. As no reliable data are available about pig importation to Hungary from

309  that period, it cannot be excluded that a wild-type 2 PRRSV was introduced elsewhere in the

310  region and then transported to Hungary.

311      Jackova et al. (2013) published a partial 432 base ORF5 sequence corresponding to the

312  ectodomain of GP5 of a Slovakian isolate from 2003 (strain 36M, acc. number: KC522648),

313  that is 95% similar at nucleotide and 97% similar at amino acid level. As the strain analyzed

314  in our study was isolated from a farm located close to the Slovakian border, and since no

315  other related strains from independent locations were present in public and private databases,

316  this virus is the most likely recent ancestor giving rise to the family of isolates including

317  PRRSV-2/Hungary/102/2012, HU12 (DQ366650) and HU21 (EF406336). The latter two

318  isolates from 2005 showed only 3% nucleotide and 1% amino acid differences in comparison

319  to PRRSV-2/Hungary/102/2012. This relatively slow rate of evolution in one of the most

320  variable part of the PRRSV genome might suggest that use of a type 1 modified live vaccine

321  used in the herd to control endemic type 1 PRRSV did not provoke a strong selective pressure

322  against the type 2 isolate. Hence, it remained conserved over a period of at least 7 years.

323      Although nsp2 insertions and deletions were present in the genome compared to

324  JAX1 and VR2332, unusual characteristics of pathogenicity or infectivity were not observed

325  under field conditions. However, exact statements on these parameters can only be given after

326    controlled challenge studies. Whether these deletions and the insertion had a negative effect

327    on tissue tropism, growth rate and/or speed of the genetic change of PRRSV-

328    2/Hungary/102/2012 further investigations are needed.

329        Antigenic regions of GP2 are relatively conserved, so it is likely that they are

330    functionally important and PRRSV does not tolerate amino acid changes in these regions. The

331    majority of amino acid changes among the investigated strains are positioned in the N-

332    terminal ($GP2_{1-40}$) and the C-terminal ($GP2_{240-256}$) regions of GP2. The N-terminal region

333    contains a hydrophobic stretch of amino acids ($GP2_{27-40}$) that has the potential to be a

334    conditional membrane binding site and/or part of a signal peptide. Although mutations are

335    common in this region, the physicochemical character of the protein remains well conserved,

336    reinforcing the putative functional significance of this site. Similar hypermutability with

337    conserved physicochemical characters can be observed in the amino-terminal hydrophobic

338    regions of all GP proteins ($GP3_{1-30}$, $GP4_{1-20}$ and $GP5_{1-31}$) and all of them are signal peptides

339    (Das et al., 2010; Thaa et al., 2013; Meulenburg, 2000; Kim et al., 2013). The N-glycan

340    addition at N184 in GP2 is critical for recovery of infectious virus but the lack of glycan in

341    N178 does not effect virus growth (Das et al., 2010). A mutation was found within the

342    glycosylation recognition sequence of N184 in PRRSV-2/Hungary/102/2012 compared to the

343    reference strains but this change most probably does not inhibit glycosylation of the site.

344        Excluding $AR_{137-159}$, the predicted B cell epitopes of GP3 are conserved. The reasons

345    for conservation might be similar to those of GP2. A previous study demonstrated that six

346    sites (Das et al., 2010) of GP3 have glycan moieties from seven potential N-linked

347    glycosylation sites, and N195 is not used for glycosylation. All glycosylation sites are present

348    on GP3 of PRRSV-2/Hungary/102/2012. Mutations are present in two motifs (N29 and N152),

349    but are not likely to affect the glycosylation status.

350        $AR_{51-65}$, a hypervariable region in GP4, is considered as neutralization epitope in type

351    1 PRRSV Lelystad virus. Previous studies have demonstrated that this epitope is susceptible

352    to monoclonal antibody-induced immunoselection *in vitro* (Costers et al., 2010), thus

353    explaining the high variability of this antigenic region. The conserved C terminal anchor

354    ($GP3_{181-197}$) of GP3 overlaps with the variable N-terminus of GP4, which serves as a signal

355    peptide and contain a T-cell epitope ($Tce_{7-15}$). Interestingly, similar overlaps can be observed

356    between the conserved GP2 C-terminal membrane anchor ($GP2_{210-232}$) and the hypervariable

357    N-terminal signal peptide of GP3. This sequence pattern suggests that the amino acids in

358    membrane anchors of GP2 and GP3 have additional sequence specific functions (e.g. protein-

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

360　conservation.

361　　　　All the four potential glycosylation sites of GP4 of VR2332 were shown to be

362　glycosylated. GP4 of PRRSV-2/Hungary/102/2012 contains a fifth N-glycosylation site (N57)

363　in the $AR_{51-65}$. It can be speculated that this potential glycosylation site might function as an

364　anchor point for glycan shielding.

365　　　　$AR_{1-15}$ within GP5 is part of signal peptide which is cleaved during peptide processing.

366　$AR_{27-35}$ may function as a decoy epitope, which is hypervariable and is not involved in

367　neutralization. However, recently it has experimentally been proved, that this epitope is only

368　present at very low frequencies as a result of an alternative cleavage site of the signal peptide

369　after the aa 26. (Ostrowski et al., 2002; Thaa et al., 2013). A previous study has demonstrated

370　that aa positions at 32, 33 and 34 of GP5 are under significant positive selection (Delisle et al.,

371　2012). $AR_{27-35}$ contains a functional glycosylation site N34 in NVSL 97-7895, while three

372　potential sites (N30 N34 and N35) can be found in the same region of PRRSV-

373　2/Hungary/102/2012. N30, N34 and N35, together with the highly conserved N44 and N51

374　compose a very rare combination of N-glycosylation sites on the GP5 of type 2 PRRSVs, less

375　than 1% of the strains contain this pattern (Delisle et al., 2012). The close proximity of N30,

376　N34 and N35 makes it improbable (because of steric inhibition) that all the three sites would

377　be glycosylated together on the same GP5 molecule of PRRSV-2/Hungary/102/2012. On the

378　other hand, considering the positive selection pressure in the region (Delisle et al., 2012), it is

379　also unlikely that the presence of the three glycosylation sites on GP5 would be just a

380　functionless arbitrary event. Most probably these sites are glycosylated in several

381　combinations on different GP5 molecules that compose a set of glycoforms in the PRRSV-

382　2/Hungary/102/2012 envelope similarly as it was shown in the VR2332 virion (Thaa et al.,

383　2013). Alternatively, it cannot be excluded that glycosylation patterns are not under selection,

384　but instead are derivative to changes in ORF5a (Robinson et al., 2013).

385　　　　As a large majority of the amino acid alterations in GP4 and GP5 of our isolate were

386　found in the previously described antigenic regions, we hypothesize that immunological

387　pressure played an important role in the evolution of the virus. It is possible that an early, fast

388　evolution period might have occurred in the early 2000's when an exponential increase in

389　genetic diversity has been observed among type 2 strains (Shi et al. 2010b). Similar strains

390　were first identified in 2005 by our research team (Balka et al., 2008), and anecdotal

391　information suggested the import of boars from Canada to the herd in previous years. The

392　comparison of these early strains and the recent isolate revealed the slowing down of the

393 genetic change as only 1% amino acid change occurred in the ORF5 ectodomain in the 7

394 years between initial sequencing and the recent viral isolation in 2012. These findings indicate

395 a biphasic evolution with a fast, early developmental stage, when the virus may have been

396 surrounded by other type 2 isolates, and a second slower phase after the virus was introduced

397 to this region, and was not influenced by immunity against other type 2 strains.

398 We conclude that PRRSV-2/Hungary/102/2012 is the first type 2 PRRSV isolated in

399 Europe that belongs to the lineage 1 or 2, and unlike every other European type 2 strain, it is

400 proved to be not related to the Ingelvac MLV. Lineages 1 and 2 were exclusively formed by

401 North American sequences until now. These data suggest that the strain was imported directly

402 from North America during the early stages of PRRSV diversification (most likely from

403 Canada or the North Central USA), and the divergent evolution of the viruses in the two

404 continents resulted in marked genetic differences among PRRSV-2/Hungary/102/2012 and

405 other type 2 viruses.

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591

592   **Figure legends**

593

594   Figure 1.

595   Molecular phylogenetic analysis of PRRSV whole genomes by maximum likelihood method.

596   Lineage designations are from Shi et al., 2010b.

597

598   Figure 2.

599   Maximum likelihood phylogenetic trees for PRRSV-2/Hungary/102/2012 ORF5 (A.)

600   designations are from Shi et al., 2010b. (B.) Representative lineage 1 and 2 sequences

601   including closest Genbank BLAST hits to PRRSV-2/Hungary/102/2012 (arrow). Closed

602   circles are all sequences with 92% sequence similarity. Open circle has 91% similarity. MB

603   CA: Manitoba, Canada; MN, Minnesota; AR, Arkansas; ?, source not provided in Genbank.

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605   Figure 3.

606   Insertion/deletion analysis of PRRSV-2/Hungary/102/2012 by the alignment to the type 2

607   prototype strain VR2332 and the Chinese highly pathogenic reference strain JXA-1.

608   (A) Whole genome sequence schematic with open reading frames (B) Expanded view of the

609   nsp2 nucleotide sequence region showing insertions and deletions (C) Amino acid sequence

610   analysis of nsp2 ORF insertion and deletion pattern.

611

612   Figure 4.

613   Alignments and analysis of envelope glycoproteins (GP) 2, 3, 4, and 5 of isolates PRRSV-

614   2/Hungary/102/2012, VR2332, Ch-1a and NVSL 97-7895. Experimentally confirmed and

615   identifiable B-cell epitopes are highlighted by grey boxes. Antigenic regions (AR) are

616   numbered by position. The overlapping regions of ARs are highlighted by dark grey boxes.

617   (A.) GP2. Experimentally confirmed glycolysation sites are underlined and set in bold (de

618   Lima et al., 2006; Vanhee et al.,2011; Das et al., 2010). (B.) GP3. Experimentally confirmed

619   glycosylation sites are underlined and set in bold (de Lima et al., 2006; Das et al., 2010; Zhou

620   et al, 2006; Wang et al., 2014). (C.) GP4. Potential glycosylation sites are underlined and

621   experimentally confirmed sites are set in bold (de Lima et al., 2006; Das et al., 2010;Diaz et

622   al., 2009; Costers et al., 2010). (D.) GP5. Antigenic regions (AR) are numbered by position. T

623   cell epitopes (Tce) are highlighted by open boxes. Potential glycosylation sites are underlined,

624   the experimentally confirmed ones are set in bold (de Lima et al., 2006; Diaz et al., 2009;

625    Plagemann et al., 2002; Ostrowski et al., 2002; Zhou et al., 2009; Mokhtar et al., 2014;

626    Vashisht et al., 2008).

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628    Table 1. Oligonucleotide primers used for RT-PCR amplification of PRRSV-

629    2/Hungary/102/2012.

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

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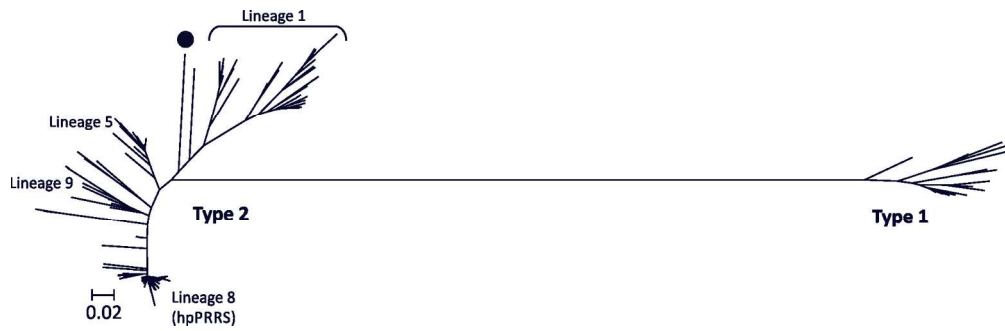
Molecular phylogenetic analysis of PRRSV whole genomes by maximum likelihood method. Lineage designations are from Shi et al., 2010b.
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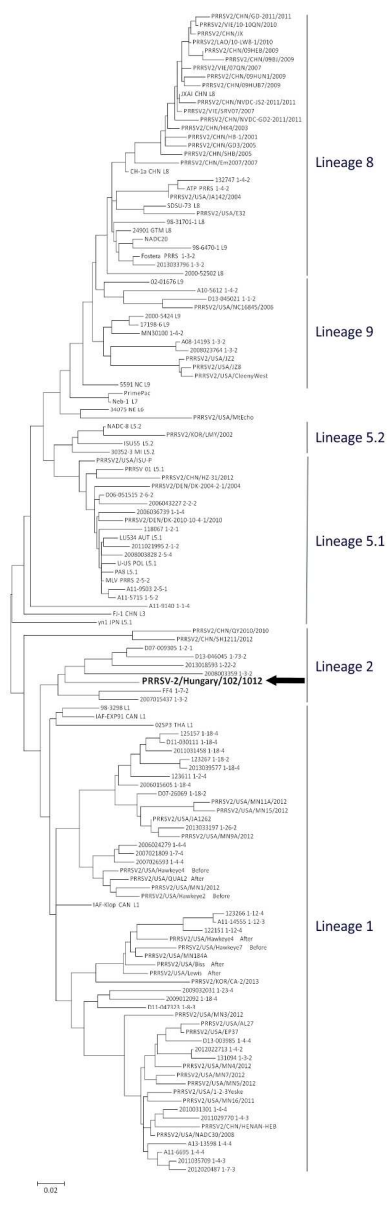


Maximum likelihood phylogenetic trees for PRRSV-2/Hungary/102/2012 ORF5 (A.) designations are from Shi et al., 2010b.
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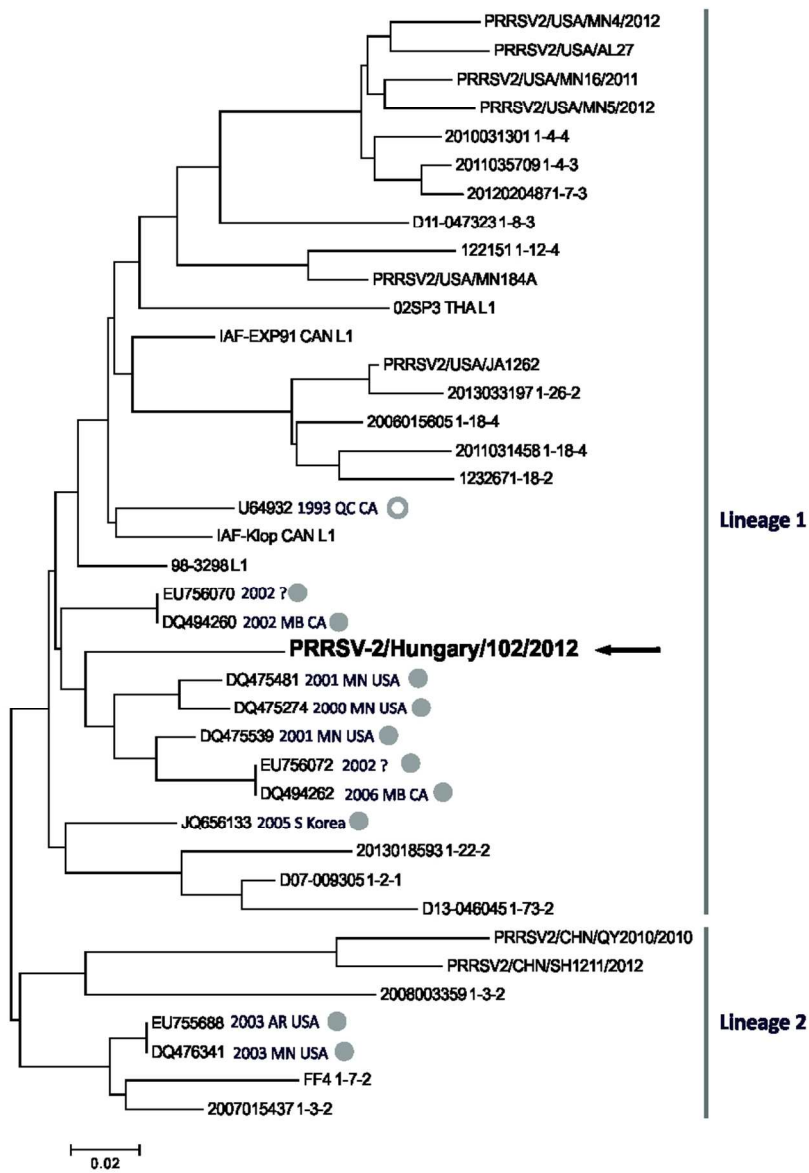


Maximum likelihood phylogenetic trees for PRRSV-2/Hungary/102/2012 ORF5 (B.) Representative lineage 1 and 2 sequences including closest Genbank BLAST hits to PRRSV-2/Hungary/102/2012 (arrow). Closed circles are all sequences with 92% sequence similarity. Open circle has 91% similarity. MB CA: Manitoba, Canada; MN, Minnesota; AR, Arkansas; ?, source not provided in Genbank.
164x229mm (150 x 150 DPI)
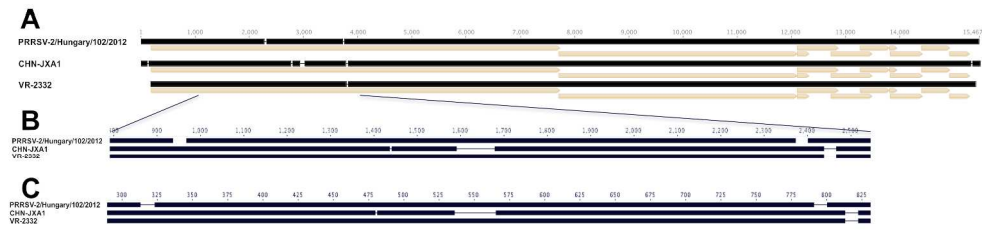
Insertion/deletion analysis of PRRSV-2/Hungary/102/2012 by the alignment to the type 2 prototype strain VR2332 and the Chinese highly pathogenic reference strain JXA-1.
(A) Whole genome sequence schematic with open reading frames (B) Expanded view of the nsp2 nucleotide sequence region showing insertions and deletions (C) Amino acid sequence analysis of nsp2 ORF insertion and deletion pattern.

258x61mm (300 x 300 DPI)
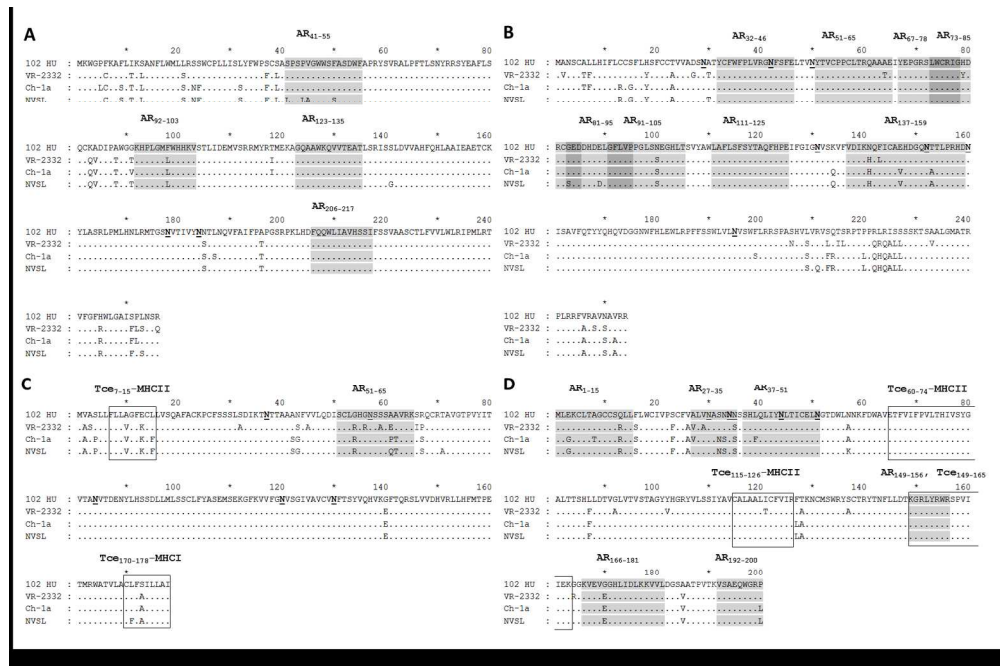
Alignments and analysis of envelope glycoproteins (GP) 2, 3, 4, and 5 of isolates PRRSV-2/Hungary/102/2012, VR2332, Ch-1a and NVSL 97-7895. Experimentally confirmed and identifiable B-cell epitopes are highlighted by grey boxes. Antigenic regions (AR) are numbered by position. The overlapping regions 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).
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| Primer | Sequence | Position |
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| 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/2009 | 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-A8/20 | 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/VR2332/1995 | 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-2010-10-4 | 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 |

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|---|---|---|---|---|
| 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 |
| PRRSV2/CHN/09SD/2009 | JF268678 | 09SD | CHN |
| PRRSV2/CHN/09BJ/2009 | JF268676 | 09BJ | CHN |
| PRRSV2/CHN/09JS/2009 | JF268675 | 09JS | CHN |
| PRRSV2/CHN/09HUN1/200 | 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/: | 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 |

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| PRRSV2/CHN/GX10-48/200 | JQ309823 | GX10-48 | CHN |
| PRRSV2/USA/NC16845/200 | HQ699067 | NC16845 | USA |
| PRRSV2/CHN/DY/2007 | JN864948 | DY | CHN |
| PRRSV2/CHN/HLJHL/2009 | HM189676 | HLJHL | CHN |
| PRRSV2/CHN/GX09-16/200 | 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/200 | 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-07/2007 | FJ349261 | KNU-07 | KOR |
| PRRSV2/CHN/SY0608/2006 | EU144079 | SY0608 | CHN |
| PRRSV2/CHN/WUH3/2008 | HM853673 | WUH3 | CHN |
| PRRSV2/CHN/08HuN/2008 | GU169411 | 08HuN | CHN |
| PRRSV2/CHN/HB-1(sh)/200 | AY150312 | HB-1(sh)/2002 | CHN |
| PRRSV2/CHN/CWZ-1-F3/20 | 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/MN184C | 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 |

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| 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_Afte | 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/SX2009/2009 | FJ895329 | SX2009 | CHN |
| PRRSV2/CHN/07BJ/2007 | FJ393459 | 07BJ | CHN |
| PRRSV2/CHN/07HEBTJ/200 | 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_Befor | 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 virus | 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 |

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| 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 |

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For Peer Review Only

| Region | Year |
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| Nanjing | 2012 |
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| Iowa | 1997 |
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| Henan | 2013 |
| Xinjiang | 2012 |
| Hubei | 2012 |
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| Yunnan | 2011 |
| Guangxi | 2010 |
| Shangai | 2008 |
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| Sichuan | 2009 |
| Guangdong | 2011 |
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| Guangdong | 2010 |
| Jiangsu | 2011 |
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| Inner Mongolia | 2009 |
| Beijing | 2006 |
| Guangdong | 2007 |
| Anhui | 2007 |
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| Hebei | 2009 |
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| Jiangsu | 2009 |
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| Sichuan | 2009 |
| Shanxi | 2009 |
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| Guangxi | 2010 |
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| Shandong | 2010 |
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| Beijing | 2010 |
| Jiangxi | 2010 |
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