Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus

Stanislav Indík,1 Lubomír Valíček,1 Dieter Klein2 and Jana Klánová3

1Veterinary Research Institute, Hudcova 70, Brno 621 32, Czech Republic
2University of Veterinary Sciences, Veterinárníplatz 1, A-1210 Vienna, Austria
3Faculty of Science, Masaryk University, Kotlářská 2, Brno 611 37, Czech Republic

The major envelope glycoprotein genes (ORF5) of seven Czech isolates of porcine reproductive and respiratory syndrome virus (PRRSV) were amplified and their nucleotide sequences were determined. ORF5 displayed nucleotide and amino acid identities of 87.5–100% and 87.6–100%, respectively, among the isolates. In a phylogenetic tree, all European isolates were grouped in a genotype distinct from that of reference American strains (VR-2332, IAF-Klop). Among the European isolates, two different clades were identified. Two Czech isolates (V-501 and V-503) and Italian strain PRRSV 2156 fell into one clade. The remaining European strains comprised the second clade. Surprisingly, two separately clustered strains (V-501 and V-516) were isolated from the same herd. Additionally, the possible effect of in vitro cultivation on the nucleotide sequence was analysed. Nine point mutations in the ORF5 region resulted from 152 in vitro passages of the V-502 isolate in MARC-145 cells.

Porcine reproductive and respiratory syndrome (PRRS) is a virus disease of pigs characterized by late-term abortions and stillbirths and by respiratory distress in nursed pigs (Wensvoort et al., 1991). The first cases were reported from North America in 1987 (Keffaber, 1989) and from Europe in 1990 (Wensvoort et al., 1991; Paton et al., 1991). The causative agent is a small, enveloped, positive-stranded RNA virus, PRRSV, classified as a member of the family Arteriviridae (Plagemann & Moenning, 1992; Meulenberg et al., 1993). The PRRSV genome is about 15 kb in length and contains eight ORFs. ORFs 1a and 1b are predicted to encode functional products associated with virus replication. Six PRRSV structural proteins, designated GP2, GP3, GP4, GP5, M and N, have been identified and found to be encoded by ORF2 to ORF7, respectively, located at the 3’ end of the genome (Mardassi et al., 1995; Meulenberg et al., 1995).

The major viral glycoprotein, GP5, binds to the M protein to form a disulphide-linked heterodimer and contains immunologically important domains associated with virus neutralization (Meulenberg et al., 1997; Pirzadeh & Dea, 1997; van Nieuwstadt et al., 1996). Pigs immunized with plasmid DNA encoding GP5 of PRRSV produced specific anti-GP5 neutralizing antibodies and induced a cellular immune response, thus protecting the vaccinated pigs from the development of viraemia and the build-up of typical macroscopic lung lesions (Pirzadeh & Dea, 1998).

Several studies have described a high degree of antigenic and genetic diversity between American and European PRRSV isolates (Meng et al., 1995; Kapur et al., 1996; Andreyev et al., 1997). GP5 has been found to be the most variable protein, with only 51–55% amino acid sequence identity between American and European isolates (Murtaugh et al., 1995; Kapur et al., 1996). ORF5 (and ORF3) also shows the highest degree of diversity within one genotype (87.1–99.2% identity among European isolates; 85–99% identity among American isolates) (Suárez et al., 1996; Pirzadeh et al., 1998).

PRRS was diagnosed in the Czech Republic by serological methods and by RT–PCR for the first time in 1995 and seven virus strains were subsequently isolated (Valíček et al., 1997, 1999). In order to determine the amount of genetic variation among these Czech isolates, the ORF5 nucleotide sequences of the seven Czech strains were determined and compared with each other and with the sequences of the European strains present in the GenBank database. In further studies, the effects on nucleotide sequences of in vitro passage of the V-502 strain were analysed.

The seven Czech PRRSV isolates used in this study were isolated in porcine alveolar macrophages in 1995 (V-503 and V-516), 1996 (V-501 and V-502) and 1998 (V-546, V-547 and V-548). Some of the isolates were adapted to grow in the MARC-145 cell line (Kim et al., 1993), which was also used as a source of high-passage virus.
Total intracellular RNA was isolated from the infected cells with Trizol LS. The ORF5 region was amplified as described previously by Oleksiewicz et al. (1998) using European type-specific primers. The forward primer 5' CAATGAGGTGG-GCTACAACC 3' and the reverse primer 5' TATGTGATG-CTAAAGGCTAGCAC 3', corresponding to nucleotides 13432–13451 and 14129–14150, respectively, of the Lelystad strain, generated a 719 bp DNA fragment. The RT–PCR products, encompassing the entire ORF5 with flanking regions from ORF4 and ORF6, were purified and both strands were sequenced on an Applied Biosystems 310 automated sequencer.

The nucleotide and deduced amino acid sequences were aligned by using CLUSTAL W (Thompson et al., 1994). Phylogenetic analysis was performed by the neighbour-joining method using the PHYLIP 3.5 software (Felsenstein, 1993). The robustness of the phylogenetic analysis and significance of branch order were determined by bootstrap analysis with 1000 replications (Felsenstein, 1985). Graphic outputs of phylogenetic trees were produced by TreeView 1.5 (Page, 1996). Hydrophobicity plots were generated by the Kyte and Doolittle method by using the Antheprot V4.0 software. The ORF5 nucleotide sequences of the Czech isolates have been deposited in GenBank under the accession numbers AF253531–AF253537.

The nucleotide and deduced amino acid sequences of the envelope glycoprotein GP5 genes of seven Czech PRRSV isolates were determined and compared with known sequences of the European and American prototype strains Lelystad and VR-2332. All of the Czech isolates were more related to the Lelystad strain than to the VR-2332 strain. No nucleotide insertions or deletions that may have resulted in amino acid insertions or deletions or frame shifting were found in the ORF5 sequences of the field isolates.

Sequences of five of the seven field isolates obtained from different herds were highly conserved and their nucleotide sequences displayed a high degree of similarity to the Lelystad strain. The among-isolate nucleotide identities ranged from 99–3 to 100%. The remaining two isolates, V-501 and V-503, shared only 88–1 and 88–9% nucleotide identity, respectively, with the European reference strain Lelystad. Nevertheless, 45–3 and 45–8% divergence was obtained when these isolates were compared with the VR-2332 strain, which is almost identical to the divergence between the Lelystad and VR-2332 strains.

Third base position mutations were predominant in ORF5. More than 60% of the mutations occurred at this last codon position and did not result in amino acid replacement, thus indicating an evolutionary stabilizing pressure to conserve the amino acid sequence.

The alignment of the deduced amino acid sequences of the Czech isolates is illustrated in Fig. 1. The envelope glycoprotein amino acid sequence for each strain consisted of 201 amino acids and the among-strain identity was 87–7–100%. The N terminus, containing the putative signal sequence, was the most variable region. Despite the high variability of the putative signal sequence, the cleavage site was situated between positions 32 and 33 for all the isolates tested. Other amino acid substitutions affected positions 56–63, 100–122, 75, 136, 140, 154, 173, 175 and 196. The Czech isolates, as well as the Lelystad strain, possessed two potential N-glycosylation sites underlined and the signal peptide is indicated by a line above the sequence.
Variations in glycoprotein GP5 of PRRSV

Fig. 2. Hydrophobicity plots of ORF5 generated by the Kyte and Doolittle method. Major areas of difference are indicated by arrows.

sites, located at amino acids 46 and 53. An additional N-glycosylation site at position 37 was predicted in isolates V-501 and V-503. The region between amino acid residues 100 and 122 has previously been reported as a conserved part of GP5 (Andreyev et al., 1997). A comparison of this region with that of the other European PRRSV isolates revealed many point mutations in this part of the protein. The membrane topology of PRRSV GP5 is not known, but the amino acid sequence and hydrophobicity profile are similar to those of the primary virion envelope glycoprotein, VP-3, of lactate dehydrogenase-elevating virus (LDV). Thus, the membrane topology of GP5 is probably similar to that of VP-3. Three closely adjacent transmembrane segments are located in the middle of the coding region of ORF5 (Faaberg & Plagemann, 1995; Meulenberg et al., 1995). At least a part of the variable region between amino acids 100 and 122 is probably situated outside the virion, between two membrane-spanning domains, where it is probably subjected to greater immunoselective pressure, thus causing variations in amino acid sequence.

Among-strain differences in glycosylation patterns can also play a considerable role. Different glycosylation patterns may be responsible for the variable immunogenicity of the proteins. This is suggested by previously published work, in which mice immunized with purified virus did not raise antibodies against the major envelope glycoprotein (Dea et al., 1996; Drew et al., 1995; van Nieuwstadt et al., 1996). On the other hand, mice immunized with the unglycosylated, E. coli-expressed ORF5 product raised antibodies against GP5 (Pirzadeh & Dea, 1997). Whether the variable glycosylation pattern of GP5 contributes to the establishment of persistent infection remains to be demonstrated.

Fig. 2 shows the GP5 hydrophobicity plots of isolates V-501 and V-503 and the Lelystad and VR-2332 strains. The separately clustered isolate V-503 had a profile similar to those of the Lelystad and VR-2332 strains. Surprisingly, the hydrophobicity plots revealed differences in the profile of the second separately clustered strain V-501. One of the differences was located between amino acids 137 and 143, where a hydrophilic peak was absent. Another variable area was noted between amino acids 53 and 60. Each of these two differences resulted from a single amino acid substitution, namely R to C (aa 140) and D to V (aa 56), respectively. The substitution D to V is probably located within the ectodomain of the protein (by analogy with VP-3 of LDV after removal of the signal peptide, approximately 30 aa in length), to which neutralizing antibodies are probably directed. A single point mutation in the epitope recognizable by an antibody may change the reactivity of the strain to antibodies. The differences between V-501 and V-503 could have resulted from evolution, but they could also have been a consequence of immune pressure against GP5 and selection of a neutralization-resistant mutant. The V-501 strain was isolated from a sentinel pig 90
days after its introduction into a PRRSV-positive herd. Therefore, this change may have led to the establishment of persistent infection. Studies with LDV have shown that only a small amount of variation within the VP-3 ectodomain can affect interactions with neutralizing antibodies, cell tropism and the maintenance of persistent infection (Chen et al., 1998).

A phylogenetic tree (Fig. 3) was constructed based on the nucleotide sequences of the incomplete ORF5 genomic regions of seven Czech strains, 19 other European strains (Suárez et al., 1996) and two American strains of PRRSV. Phylogenetic analysis generated by the neighbour-joining method divided the European strains into two clades. Two Czech isolates (V-501 and V-503) and the Italian PRRSV 2156 strain formed one subgroup and the remaining European isolates comprised another subgroup. The robustness of this grouping was supported by a very high bootstrap value (100%) obtained from bootstrap resampling analysis with 1000 replications. The remaining five Czech isolates were grouped within a broad clade, together with the Lelystad strain. Strain VR-2332 always clustered apart from the European isolates when the nucleotide sequence from the corresponding region was included in this analysis.

This study documents significant diversity among the Czech isolates. It is noteworthy that two different strains (V-516 and V-501) were isolated from the same herd, in 1995 and 1996. This was probably caused by the introduction of a new variant into the herd rather than by local evolution, because different variants of PRRSV were also identified at the same time (V-503 and V-516 in 1995; V-501 and V-502 in 1996) on other farms in the Czech Republic. Moreover, the period of 1 year is probably not sufficient for such an evolutionary effect. Goldberg et al. (2000), who also reported different variants in a single herd, suggested that the within-herd genetic variability was caused by transport of animals or semen rather than by local evolution.

The nine point mutations that resulted from 152 in vitro passages of the V-502 isolate in cell culture at 37 °C appeared to be scattered randomly across the protein. Five of them resulted in amino acid substitutions (R to C, L to F, L to F, V to I and R to G at positions 9, 20, 71, 113 and 153). Such a high mutation rate is expected for RNA viruses. These amino acid substitutions did not affect the ectodomain of the glycoprotein, but two mutations were detected within the signal peptide. An arginine at residue 153 was replaced by the uncharged amino acid glycine, which is also present at the same position of ORF5 in the vaccine strain RespPRRS, derived by attenuation of the VR-2332 strain in MARC-145 cells. This mutation changes the polarity of the hydrophilic region of the endodomain and could have been one of the mutations that resulted in a decrease in virulence. Unfortunately, this mutation reverts rapidly as the virus replicates in pigs (Madsen et al., 1998; Wesley et al., 1999).

In summary, our results demonstrate high variability among Czech PRRSV isolates. Moreover, the isolation of two distinct virus variants on a single farm over the period of 1 year is strongly suggestive of their co-existence. This may represent
an additional consideration in the development of effective control strategies and in vaccine development.

We wish to thank A. Cepica, I. Stepanek and B. Salmons for their contribution to the improvement of the text and their excellent recommendations. This work was supported by the Ministry of Agriculture and by the Grant Agency of the Czech Republic (project no. EP 9186; grant no. 508/95/0377).

References


Chen, Z., Li, K., Rowland, R. R. & Plagemann, P. G. (1998). Neuropathogenicity and susceptibility to immune response are interdependent properties of lactate dehydrogenase-elevating virus (LDV) and correlate with the number of N-linked polylactosaminoglycan chains on the ectodomain of the primary envelope glycoprotein. Advances in Experimental Medicine and Biology 441, 583–592.


Received 18 April 2000; Accepted 14 June 2000