# Differentiation of porcine reproductive and respiratory syndrome virus European vaccine strains from Czech field isolates by restriction fragment length polymorphism analysis of ORF5 gene

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**ABSTRACT**: Restriction fragment length polymorphism (RFLP) analysis of open reading frame 5 was developed for typing of Czech strains of porcine reproductive and respiratory syndrome virus (PRRSV). The set of restriction enzymes *Acc* I, *Hae* II and *Sna*B I allowed the differentiation of heterogeneous Czech strains of PRRSV clustered separately in the phylogenetic tree. The high-passage strain V-502 (164) was also differentiated from its parent strain V-502. The same restriction enzymes could distinguish the European-type vaccine strains Porcilis PRRS and Pyrsvac-183, registered in Czech Republic, from the Czech field isolates. The published ORF5 nucleotide sequences allowed us to presume that it will also be possible to distinguish most of European field strains from vaccine strains. PCR-based RFLP analysis can become a valuable tool in epidemiological studies of PRRSV in Europe.

**Keywords**: RFLP; PRRSV; porcilis; pyrsvac; ORF5

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pig pathogen classified, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV), in the genus *Arterivirus*, family Arteriviridae, order *Nidovirales*, the latter including also the family Coronaviridae (Cavanagh, 1997). PRRS is manifested by reproductive failures in sows and respiratory distress in piglets.

The causative agent of the disease was identified first in The Netherlands in 1991 (Wensvoort *et al.*, 1991). The RNA genome is a single-stranded positive sense molecule approximately 15 kb in length which contains eight open reading frames (ORFs). The first two, ORF1a and ORF1b, encode proteins with polymerase and replicase activities. ORF2–ORF7 encode structural proteins of PRRSV.

PRRSV isolates exhibit a high degree of genetic variability. European and American genotypes show only 55–80% nucleotide identity (Meng *et al.*, 1995; Murtaugh *et al.*, 1995; Suarez *et al.*, 1996; Meulenberg *et al.*, 1997). Moreover, significant within-genotype differences in nucleotide and amino acid sequences have been demonstrated. Monoclonal antibodies (MAb) can be used to group PRRSV strains into two genotypes (Murtaugh *et al.*, 1995). Moreover, the two genotypes can be differentiated by RT-PCR (Mardassi *et al.*, 1994; Oleksiewicz *et al.*, 1998).

Significant differences in nucleotide sequences among Czech European-type isolates of PRRSV were found in our previous study by identification of two separately clustered groups of PRRSV (Indik *et al.*, 2000). One group with nucleotide sequences displayed a high degree of similarity to the European reference strain Lelystad. The other group clustered in the phylogenetic tree separately from all European isolates of PRRSV (except for the Italian strain PRRSv 2156).

A commercial modified live European-type vaccine strains has been used to immunise pigs on Czech farms. Like virulent strains, attenuated

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vaccine strains of PRRSV, can persist in vaccinated pigs and be shed for several months.

ORF5 PCR-based restriction fragment length polymorphism (RFLP) analysis has been developed for the differentiation of American PRRSV strains (Wesley *et al.*, 1998, 1999). Amplified ORF5 region was digested with restriction endonucleases *Mlu* I, *Hinc* II and *Sac* II. This digestion allowed the differentiation of the vaccine RespPRRS and parent virus VR-2332 from other American PRRSV strains. Cheon and Chae (2000) completed the set of enzymes with the restrictase *Hae* III to differentiate American-type viruses isolated in Korea. Because of a high degree of nucleotide sequences variability between the American and the European strains of PRRSV, these enzymes could not be used for RFLP analysis of European strains of PRRSV.

The aim of this study was to select a set of restriction enzymes for differentiation of two heterogeneous groups of PRRSV present on Czech farms as well as for discrimination of European-type vaccine strains registered in the Czech Republic from field isolates.

## MATERIAL AND METHODS

Three European live attenuated PRRSV vaccine strains (Amervac-PRRS/A3-Laboratories Hipra, Spain; Porcilis PRRS-Intervet, The Netherlands; Pyrsvac-183 , SYVA Laboratories, Spain ) and seven Czech strains were used for RFLP analysis and differentiation. Strain designations, years of isolation, and passage histories of the Czech PRRSV strains are given in Table 1.

The PRRSV reference strain Lelystad, the Danish isolate DK111/92, and the Slovak isolate VOS 7573 were kindly provided by dr. Wensvoort (ID Lelystad, The Netherlands), dr. Botner of the Danish Veterinary Institute, Lindholm Denmark, and dr. Mojžíš of the State Veterinary Institute, Zvolen, Slovak Republic, respectively.

Total RNA was extracted from infected cells with the Trizol LS reagent (Gibco BRL) according to the manufacturer's instructions and used as template for RT-PCR. The primers amplifying entire ORF5 have been described by Suarez *et al.* (1996). Reverse transcription and DNA amplification were com-

Table 1. Designation, origin, passage history, and RFLP code of PRRSV strains

| Virus designation | Origin | Virus isolated (year) | Passage history | | RFLP code |
|---|---|---|---|---|---|
| | | | PAM * | MARC145 | |
| CAPM V-502 | Czech Republic | 1996 | 3 | 0 | 4-3-2 |
| CAPM V-502 (164) | Czech Republic | 1996 | 4 | 160 | 1-3-2 |
| CAPM V-516 | Czech Republic | 1995 | 1 | 0 | 4-3-2 |
| CAPM V-546 | Czech Republic | 1998 | 3 | 0 | 4-3-2 |
| CAPM V-547 | Czech Republic | 1998 | 4 | 0 | 4-3-2 |
| CAPM V-548 | Czech Republic | 1998 | 3 | 0 | 4-3-2 |
| CAPM V-501 | Czech Republic | 1996 | 2 | 0 | 2-1-1 |
| CAPM V-503 | Czech Republic | 1995 | 4 | 0 | 2-1-1 |
| VOS 7573 | Slovak Republic | 1996 | unk** | unk | 5-5-1 |
| Lelystad | The Netherlands | 1991 | 7 | 12 | 4-3-2 |
| DK 111/92 | Denmark | 1992 | unk | 4 | 1-2-1 |
| Amervac-PRRS/A3 | Spain | vaccine strain | unk | unk | 1-4-1 |
| PYRSVAC-183 | Spain | vaccine strain | unk | unk | 1-4-1 |
| Porcilis PRRS | The Netherlands | vaccine strain | unk | unk | 3-3-1 |

*porcine alveolar macrophages, **unknown number of passages

bined in a single 0.2 ml PCR reaction tube using Titan One tube RT-PCR kit (Roche).

The reaction mixture contained 400 nM of each primer in the final volume of 50 µl. The conditions for RT-PCR included incubation at 50°C for 30 min and at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 68°C for 1 min. PCR was finished by hold at 68°C for 7 min. After amplification, the PCR products were purified with the PCR purification kit (Qiagen) and digested with the restriction enzymes *Acc* I, *Hae* II and *Sna*B I (New England Biolabs). The digested fragments were analysed by electrophoresis on 2% agarose gel. Different types of RFLP patterns were assigned specific numerical codes for each restriction enzyme. The ORF5 nucleotide sequences obtained previously (Indik *et al.*, 2000) or from GenBank database were aligned by using CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic tree was produced using the PHYLIP 3.5 (Felsenstein, 1993) software.

## RESULTS

Based on nucleotide sequences of ORF5 of European isolates (Suarez *et al.*, 1996; Indik *et al.*, 2000) obtained from the GenBank database and sequences identified by sequence analysis of PRRSV strains, we selected the restriction enzymes, *Acc* I, *Sna*B I and *Hae* II for RFLP analysis. The 606 bp RT-PCR products (entire ORF5) were digested and analysed by electrophoresis in agarose gel. Each isolate was assigned a numerical code for its ORF5 RFLP pattern with the enzymes *Acc* I, *Sna*B I, and

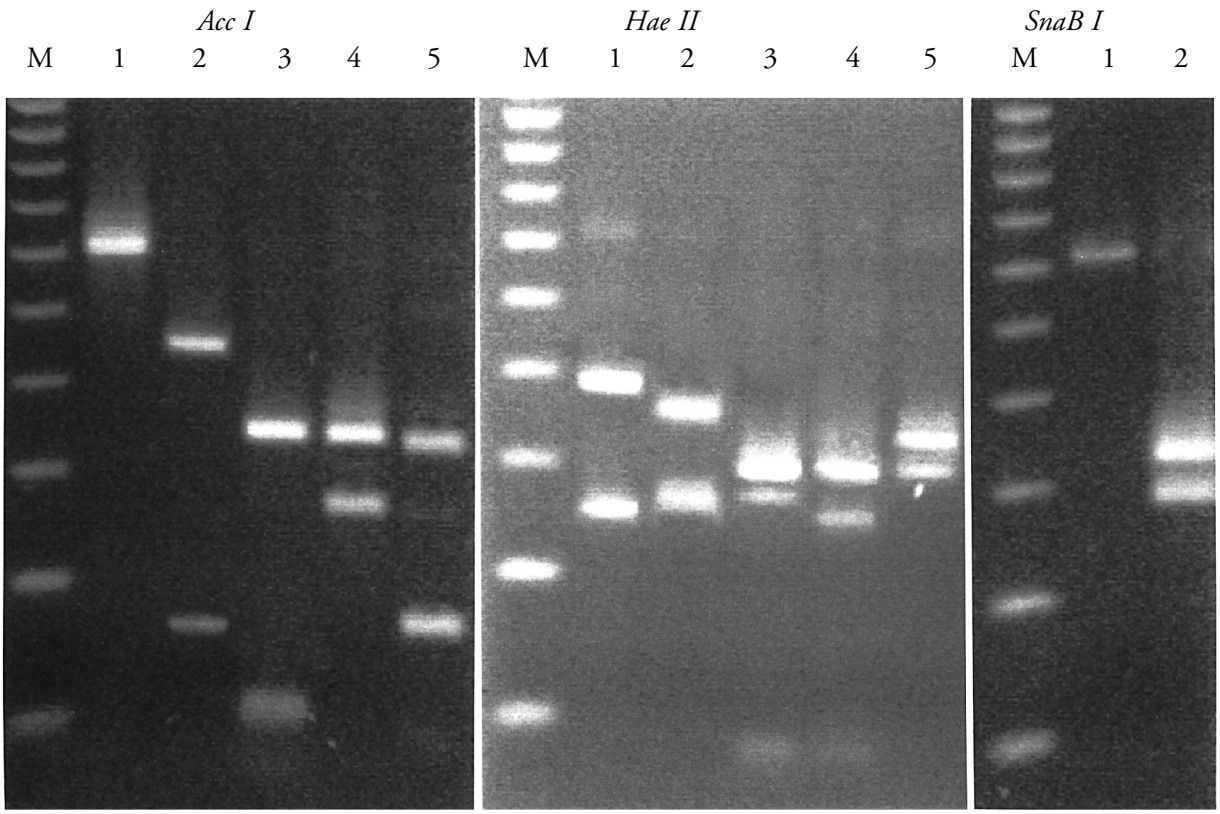


Figure 1.General types of RFLP patterns for PRRSV. 606 bp long PCR products (complete ORF5) were digested with restriction enzymes *Acc* I, *Hea* II and *SnaB* I and electrophoresed on 2% agarose gel containing ethidium bromide. Based on determined nucleotide sequences of PRRSV isolates the length of fragments was predicted: *Acc* I – code 1 (606 bp), code 2 (443 bp,163 bp), code 3 (338 bp, 105 bp, 94 bp, 69 bp), code 4 (338 bp, 268 bp), code 5 (338 bp, 174 bp, 94 bp); *Hae* II – code 1 (364 bp, 242 bp), code 2 (347 bp, 259 bp), code 3 (290 bp, 259 bp, 57 bp), code 4 (290 bp, 242 bp, 57 bp, 17 bp), code 5 (316 bp, 290 bp); *SnaB* I – code 1 (606 bp), code 2 (324 bp, 282 bp). Fragments smaller than 100 bp are poorly visible. M = 100 bp low ladder (Sigma)

*Hae* II. Digestion with *Acc* I yielded five cutting patterns including a no cut pattern, designated as code 1. Digestion with *Hae* II yielded five cutting patterns and *Sna*B I either did not cut (code 1) or did cut (code 2). The cutting patterns and expected length of fragments are shown in Figure 1.

The PRRS vaccine strain Porcilis, registered in the Czech Republic, has three *Acc* I recognition sites in ORF5 at positions 337, 442 and 511, respectively. The *Acc* I cutting pattern (code 3) distinguishes the vaccine strain from field isolates. PCR products of Amervac PRRS/A3 and Pyrsvac-183 vaccine strains were not cut by *Acc* I (code 1). No cut profiles were also obtained from the high-passage strain V-502 (164) and the Danish strain DK111/92. The Czech field isolates V-502, V-516, V-546, V-547, and V-548, which are closely related to the to EU reference strain Lelystad showed a code 4 cutting pattern with the length of digested fragments 338 bp and 268 bp. Digestion with *Acc* I of the other Czech isolates V-501 and V-503, clustered separately in the phylogenetic tree, yielded a code 2 RFLP pattern (two fragments: 443 bp, 163 bp).

Further differentiation was done using the *Hae* II enzyme, which yielded five cutting patterns. The Amervac PRRS/A3 and Pyrsvac-183 vaccine strains were distinguished from DK111/92 with which they shared the *Acc* I cutting profile. The *Hae* II pattern for these vaccine strains was code 4 (three recognition sites, expected length of fragments: 290 bp, 242 bp, 57 bp and 17 bp), while *Hae* II cuts DK111/92 strain yielding code 2 pattern (length of fragments: 347 bp and 259 bp). *Hae* II also allowed differentiation within the heterogeneous group of the Czech isolates. The isolates V-502, V-516, V-546, V-547, V-548 shared the code 3 pattern

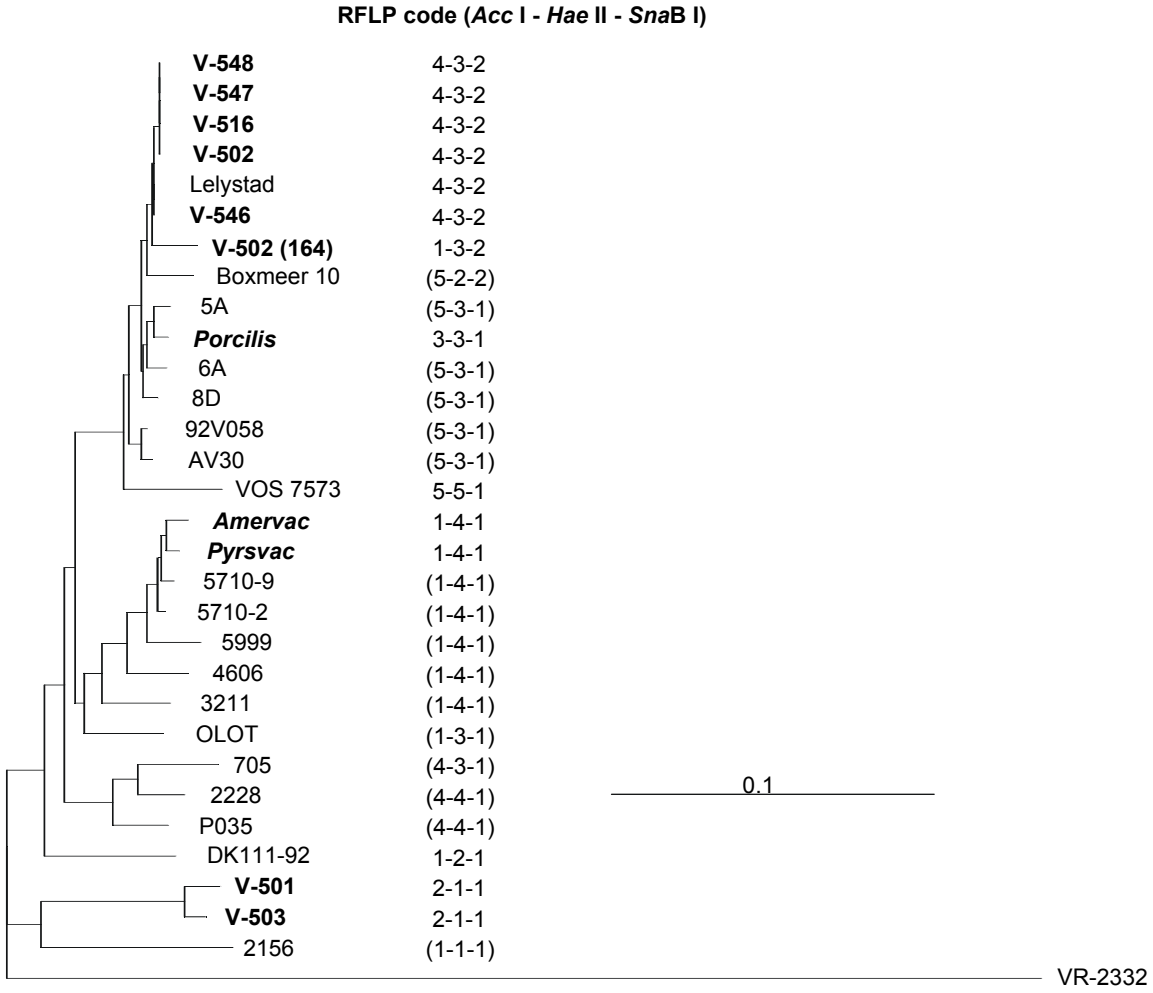


Figure 2. Phylogenetic tree based on PRRSV ORF5 nucleotide sequences and restriction fragment length polymorphism (RFLP) codes for PRRSV strains. Predicted RFLP codes from ORF5 nucleotide sequences of EU PRRSV strains present in GenBank database are given in parentheses

(fragments of 290 bp, 257 bp and 57 bp in length) with the high passage strain V-502 (164) and code 1 pattern (364 bp, 242 bp fragments) was found for the isolates V-501 and V-503.

*Sna*B I cut Porcilis PRRS as well as Pyrsvac-183 and Amervac PRRS/A3 yielding the code 1 pattern. Like in the previous case, digestion with this enzyme could distinguish among the heterogeneous Czech isolates. While the isolates V-501 and V-503 were not cut (code 1), the isolates V-502, V-516, V-546, V-547, V-548 were cut (code 2) yielding two fragments (324 bp and 282 bp).

The *Acc* I site in ORF5 at position 442 of the vaccine strain Porcilis PRRS distinguished the vaccine strain from the PRRSV field isolates. The Czech field isolates could be distinguished from the Porcilis vaccine strain also by analysing the combined *Hae* II and *Sna*B I RFLP profiles.

The Amervac-PRRS A/3 and Pyrsvac-183 strains differ from the Czech strains by their *Hae* II cutting patterns. Differentiation of these two vaccine strains from the Czech field isolates as well as from high-passage strain V-502 (164) was also possible by combining the *Acc* I and *Sna*B I cutting patterns. All the three restriction enzymes could distinguish the two groups of Czech strains clustered in the phylogenetic tree separately. While the strains of one group (V-502, V-516, V-546, V-547, V-548) yielded the *Acc* I – *Hae* II – *Sna*B I code 4-3-2 pattern, the other group (V-501 and V-503) yielded the RFLP cutting profile 2-1-1.

## DISCUSSION

Results of the current serological methods only indicate whether individual animals or the whole herd have come into contact with PRRSV, but cannot distinguish between vaccinated animals and those infected by a wild strain. As both attenuated vaccine strains and wild strains can persist in vaccinated animals for several months, a test that would differentiate between infections by wild-type strains and treatment with vaccine strains would be very useful. Wesley *et al.* (1998) developed a procedure for restriction fragment length polymorphism (RFLP) analysis that allows the differentiation of the American vaccine strain RespPRRS from most wild strains of the American genotype. High degree of genetic heterogeneity between American and European strains of PRRSV do not allow using American-type vaccines in Europe. Therefore EU-type vaccines are used in some swine herds in the Czech Republic. The differentiation between the European vaccine strains and wild strains is possible using less practical methods, such as sequencing or hybridization. A simple alternative to these sophisticated techniques is RFLP analysis.

Digestion of the vaccine strain Porcilis PRRS yielded a unique *Acc* I cutting profile (code 3) distinguishing it from all the PRRSV strains used within this study. Considering the published sequences of other European strains of PRRSV we presume that it will be possible to distinguish this vaccine strain also from the strains NL-3, Boxmeer-10, PRRSV 5A, PRRSV 6a, PRRSV 8D, PRRSV 92V058, PRRSV AV30, PRRSV 5710, PRRSV 5999, PRRSV 4606, PRRSV 3211, PRRSV OLOT, PRRSV 705, PRRSV 2228, PRRSV P035, and PRRSV 2156 (Figure 2).

The failure of *Acc* I to digest the vaccine strains Amervac-PRRS/A3 and Pyrsvac-183 (code 1), allowed us to differentiate them from the Czech field isolates. Unlike the low-passage strain V-502, its high passage variant V-502 (164) was not digested by this restriction endonuclease. The high-passage strain showed an A to G substitution at the position 337 which apparently prevented *Acc* I from recognising the site. This substitution was not identical with that in the vaccine strains Amervac-PRRS/A3 a Pyrsvac-183, which showed a C to G mutation at the position 339 (unpublished results).

*Acc* I also failed to digest the Danish strain DK111/92. The published ORF5 sequences allow us to presume that the Spanish strains PRRSV 5710, PRRSV 5999, PRRSV 4606, PRRSV 3211, and PRRSV OLOT and the Italian strain PRRSV 2156 will also share the non-cutting code 1 RFLP profile.

The Danish isolate DK111/92 was distinguished from the vaccine strains by its *Hae* II RFLP profile (code 2). Code 1 RFLP pattern was predicted for the Italian isolate PRRSV 2156. The Spanish strains PRRSV 5710, PRRSV 5999, PRRSV 4606 and PRRSV 3211 could not be differentiated from the vaccine strains Amervac-PRRS/A3 and Pyrsvac-183, because of a very close similarity of their nucleotide sequences.

The sensitivity of RT-PCR is comparable to virus isolation (Oleksiewicz *et al.*, 1998; Spagnuolo-Weaver *et al.*, 2000). Moreover, virus isolation is time consuming and difficult to perform. In case of simultaneous presence of different viral strains in clinical samples, one of them could quickly

predominate during replication in a cell culture as it was reported by Mengeling *et al.* (1999). He showed that an attenuated vaccine strain might predominate during a single passage in a cell culture. In such a case the presence of a virulent strain can be disguised. Amplification of viral RNA directly from clinical samples allows omitting virus isolation and propagation. Single additional step, RFLP analysis, is a simple and fast method, which could help to characterise PRRSV present in the clinical samples.

Results of PCR-based RFLP analysis of PRRSV isolates will be usable in epidemiological studies of PRRSV in Czech herds. The Czech field strains isolated so far can be distinguished from attenuated vaccine strains of the European genotype used for the protection of local swine herds by the RFLP pattern code for ORF5. Except for the Spanish strains (PRRSV 5710, PRRSV 5999, PRRSV 4606, PRRSV 3211, PRRSV OLOT), the RFLP analysis described here allows also differentiation between European field strains of PRRSV and vaccine strains of the European genotype.

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