Restriction fragment length polymorphism of ORF6 and ORF7 genes of porcine reproductive and respiratory syndrome virus (PRRSV) vaccine strains registered in the Czech Republic

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ABSTRACT: Restriction fragment length polymorphism (RFLP) of open reading frames 6 and 7 was applied to comparative genetic analysis of live attenuated vaccine strains (Amervac-PRRS/A3, Porcilis PRRS, Ingelvac PRRS) of porcine reproductive and respiratory syndrome virus (PRRSV), registered in the Czech Republic, six field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), and three PRRSV local field isolates (CAMP V-502, CAMP V-503, VOS 2878) found in pig herds in the Czech Republic and Slovak Republic. The set of restriction enzymes Hae II, Alu I and BsaJ I allowed the differentiation of local field isolates, field viruses of PRRS, and vaccine strains of the European genotype from North American genotype, but could also distinguish between viruses of the same genotype. Five different RFLP patterns were obtained from twelve examined PRRS viruses by combination of the above restriction enzymes. RFLP code 1-1-1 was the most frequent digestion pattern within all PRRS field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), CAMP V-502 isolate and vaccine strain Porcilis PRRS, which is suggestive of higher antigenic identity among the compared viruses. In the North American types (Ingelvac PRRS vaccine strain and VOS 2878 isolate), homogeneity in restriction patterns (code 2-x-4) was recorded. These studies indicate that PCR-based RFLP analysis of ORF6 and ORF7 of genes might be a suitable tool in epidemiological studies of PRRSV, similarly to the studies based on genetic analysis of ORF5 gene.

Keywords: RT-PCR; RFLP; PRRSV; ORF6 and ORF7; vaccine

Porcine reproductive and respiratory syndrome (PRRS) is a relatively new disease characterized by reproductive failures in sows and respiratory distress in pigs of all age categories. Infection of sows often results in late term abortions, stillbirth, mummified and weak live born pigs, which die showing the signs of muscle tremor. Clinical signs of neonatal pigs include respiratory distress, dysorexia, fever, diarrhoeas, tremor and anorexia. Mortality can be as high as 100%. Infected boars show decreased quality of semen, morphological abnormalities and decrease mobility of sperms (Goyal, 1993; Mengeling et al., 1994).

Porcine reproductive and respiratory syndrome virus is an important pathogen with huge impact on the global pig industry. The virus is a member of the genus Arterivirus, family Arteriviridae, order Nidovirales. Viral genome is formed of a single-stranded positive sense polyadenylated RNA molecule of 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 (Mengeling et al., 1994; Cavanagh, 1997). ORFs 2, 3, 4 and 5 encode structural proteins. ORF6 encodes matrix (M) protein of 18 to 19 kD and ORF7 encodes nucleocapsid (N) protein of 14 to 15 kD (Meulenberg et al., 1995).

Viral aetiology of PRRS was for the first time determined in the Netherlands in 1991 by isolation

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of the prototype strain Lelystad, and in the U.S.A. in 1992 by isolation of the prototype of American strain VR-2332 (Wensvoort et al., 1991; Collins et al., 1992). Based on comparison of nucleotide sequences of these different strains, the European and North American strains were classified into two different genotypes. These two genotypes exhibited only 55% to 80% nucleotide identity (Meng et al., 1995; Murtaugh et al., 1995; Suarez et al., 1996; Meulenberg et al., 1997). High level of genetic variability has also been described within the American group of PRRSV isolates (Andreyev et al., 1997). Until recently, the European type isolates have been considered as less variable. However, the latest studies show a significant genetic heterogeneity within the European type isolates (Indik et al., 2000; Stadejek et al., 2002). Radically different variants of PRRSV in Eastern Europe support the hypothesis that the European and American genotypes of PRRSV evolved from a common ancestor (Stadejek et al., 2002).

Vaccination of herds against PRRSV is a common practice used for protection and control of clinical signs of the infection. At present, the commercially produced vaccines are modified live vaccines (MLV) or inactivated vaccines. In some countries, autogenic inactivated vaccines prepared from a field virus have been used. Inactivated vaccines are considered to be safer but the onset of protective effect can only be seen after repeated vaccination. Live vaccines offer sufficient protection against challenge with a homologous virus, however, the vaccination effect might be limited due to great genetic variability. Live vaccination virus can also penetrate through placenta, thus being a cause of congenital infection (Mengeling et al., 1996) or can be for a certain time shed into the environment through secretion and excretion (Christopher-Hennings et al., 1997). In spite of these facts, live vaccines are predominantly used in herds with acute form of infection exhibiting clinical signs.

A modified live vaccine Ingelvac PRRS MLV (Boehringer Ingelheim Vetmedica) is considered as safe and effective tool for prevention of clinical signs, viraemia and leucopenia, which are associated with the respiratory form of pig infection. Although the vaccine has been developed from the American strain of the PRRS virus (ATCC VR-2332), cross protection against the European strain "Lelystad" has been demonstrated (Gagnon and Dea, 1998). Rossow et al. (1999) described that some strains, capable to induce infection, persist in herds in spite of application of the modified live vaccines against PRRSV. The vaccine strains can revert to the original virulence as a result of mutation. It was confirmed that distribution of the American type PRRSV in Denmark was due to introduction of vaccination by modified live virus (Madsen et al., 1998; Botner et al., 1999). The vaccine virus has higher protective efficacy against homologous strains (Labarque et al., 2004).

PRRS infection was demonstrated in pigs in the Czech Republic in 1995 and at the same time the RT-PCR method for detection of viral nucleic acid in blood serum of pigs and semen of breeding boards (Valicek et al., 1997; Psikal et al., 1998) was introduced. PCR-based restriction fragment length polymorphism (RFLP) analysis has been developed for the differentiation of PRRSV isolates (Gagnon and Dea, 1998; Wesley et al., 1998). PCR enables reproduction of small number of copies of a specific target DNA sequence and RFLP is used in molecular biology for differentiation between DNA and cDNA using restriction enzymes. The difference of the restriction sites offers an important genetic tool for diagnosis and epidemiology of the pathogen.

The objective of our study was to determine the range of genetic variability within the available vaccine strains registered in the Czech Republic, and PRRSV isolates using the RFLP method in regions of ORF6 and ORF7 genes.

MATERIAL AND METHODS

PRRSV samples

Three live attenuated vaccine strains (Porcilis PRRS, Intervet International, the Netherlands; Amervac-PRRS/A3, laboratory Hipra, Spain; Ingelvac PRRS, Boehringer Ingelheim Vetmedica, Germany), three local field isolates (CAMP[1] V-502, CAMP V-503, VOS 2878), and field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791) were used for RFLP analyses and differentiation of viruses. The field isolate VOS 2878 was isolated in 1996 from imported pigs in Slovak Republic. The

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Table 1. Designation, genotype, origin and RFLP codes of PRRSV

Virus designation	Genotype	Origin	RFLP code		
			Hae II	Alu I	BsaJ I
L-588	EU	Czech Republic	1	1	1
L-1606	EU	Czech Republic	1	1	1
L-2053	EU	Czech Republic	1	1	1
L-3305	EU	Czech Republic	1	1	1
L-6558	EU	Czech Republic	1	1	1
L-6791	EU	Czech Republic	1	1	1
CAMP V-502	EU	Czech Republic	1	1	1
CAMP V-503	EU	Czech Republic	1	3	3
VOS 2878	NA	Slovak Republic	2	5	4
Amervac-PRRS/A3	EU	Spain	1	2	2
Porcilis PRRS	EU	The Netherlands	1	1	1
Ingelvac PRRS	NA	Germany	2	4	4

EU – European genotype of the virus; NA – North American genotype of the virus

viruses are shown in Table 1 with description of their genotype and origin.

Isolation of genomic RNA from cell culture

Vaccine strains and local field isolates were propagated on cell line MARC-145. The cells were cultured in Eagle's minimal essential medium supplemented with 10% bovine fetal serum at 37°C. PRRS viruses were harvested after expression of the cytopathic effect (CPE) in 70% to 80% of cells. The total RNA was extracted from infected cells using Trizol LS (GIBCO BRL) according to the manufacturer's instructions.

Reverse transcription – polymerase chain reaction (RT-PCR)

The extracted RNA was used as a template for RT-PCR of each PRRSV strain. RT-PCR was carried out using Titan^{TM} One Tube kit (Roche). Two 20-base oligonucleotides were used as primers in RT-PCR reaction designed from a part of ORF6 and the whole ORF7 genes (Suarez et al., 1996). Forward and reverse primers were synthesized as follows: 5'-GTACATTCTGGCCCCTGCCC-3' and 5'-TCGCCCTAATTGAATAGGTG-3'.

50 µl RT-PCR reaction mixture contained 2 µl of the isolated RNA; 5 × RT-PCR reaction buffer (Roche); 5mM DTT; 0.2mM of each dNTP; 0.4µM of each primer; 40 U RNasin (Promega); 1.5mM $MgCl_2$ and 1 µl of mix-enzyme. The employed PCR profile contained thermocycler adjustment to the following programmes: cDNA synthesis at 50°C for 30 min, inactivation of reverse transcriptase and denaturation at 95°C for 5 min. This was followed by 34 cycles of denaturation at 94°C for 30 s, annealing of primers at 50°C for 1 min and extension at 68°C for 1 min. The final step included extension at 68°C for 7 min and holding the samples at 4°C.

Restriction fragment length polymorphism (RFLP)

After amplification, PCR products were purified using a PCR purification kit (Qiagen) and digested with restriction enzymes Hae II, Alu I and BsaJ I (New England BioLabs) at 37°C for 2 h (except for BsaJ I which was used at 60°C) in corresponding buffer solutions. Analysis of the restriction enzyme digestion was performed on 2% agarose gels stained with ethidium bromide or on 12.5% polyacrylamide gels stained with silver nitrate.

RESULTS

PRRSV isolation

All PRRSV strains and isolates used in this study were successfully adapted and propagated on cell line MARC-145 except for six field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791) whose RNA was directly used in RT-PCR.

RT-PCR and RFLP analysis

The use of a standard protocol, described in the Methods, allowed us to amplify the target fragment from the virus genome and to obtain PCR products of 647 bp and 670 bp for the European and the North American types, respectively (Figure 1). The obtained products were digested with selected restriction enzymes. The enzymes were selected based on sequence analysis ORF6 and ORF7 genes of PRRSV strains for which sequences from the GenBank database had been used. PCR products of twelve PRRS viruses which were exposed to RFLP analysis exhibited two, five and four different digestion RFLP patterns obtained using the enzymes Hae II, Alu I and BsaJ I, respectively.

Different types of RFLP patters were assigned specific numerical codes for each restriction enzyme, to make the analysis of the obtained data easier. These RFLP patterns are listed in Table 1. None of the selected enzymes was assigned a RFLP pattern that would contain a non-digestion profile (Wesley et al., 1998).

Digestion with Hae II yielded only two different digestion RFLP patters designated as code 1 and 2 (Table 1). The use of this restriction enzyme was important for differentiation of PRRS viruses according to their length of restriction fragments to European genotype (339 bp, 308 bp) and North American one (454 bp, 216 bp) as shown in Figure 2a. Samples of the European genotype with code 1 pattern included in our study all field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), local field isolates (CAMP V-502, CAMP V-503), and vaccine strains (Amervac-PRRS/A3, Porcilis PRRS). The remaining North American vaccine strain Ingelvac PRRS and isolate VOS 2878 were assigned to code 2.

Five distinguished digestion patterns of the enzyme Alu I were assigned to code 1–5 (Table 1). This enzyme had the same digestion pattern (code 1) in all field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), isolate CAMP V-502, and vaccine strain Porcilis PRRS with the length of restriction fragments 410 bp, 161 bp and 76 bp, respectively (Figure 2b). In the others of the group of European type virus, different digestion patterns were detected for Amervac-PRRS/A3 (code 2) and CAMP V-503 (code 3). Code 4 and 5 was assigned to the North American strain Ingelvac PRRS and isolate VOS 2878, respectively, which are different not only within this group but also in comparison with the group of European type virus.

Digestion with the last restriction enzyme BsaJ I yielded four different digestion patterns, which are shown in Table 1. The digestion pattern BsaJ I was identical in all field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), isolate CAMP V-502, and vaccine strain Porcilis PRRS of the group of European type virus, was assigned to code 1 and fragment length was 315 bp, 283 bp and 49 bp (Figure 3). The vaccine strain Amervac-PRRS/A3 and isolate CAMP V-503 had different restriction sites of the enzyme BsaJ I (codes 2, 3). Comparison of RFLP code 4 in North American vaccine strain

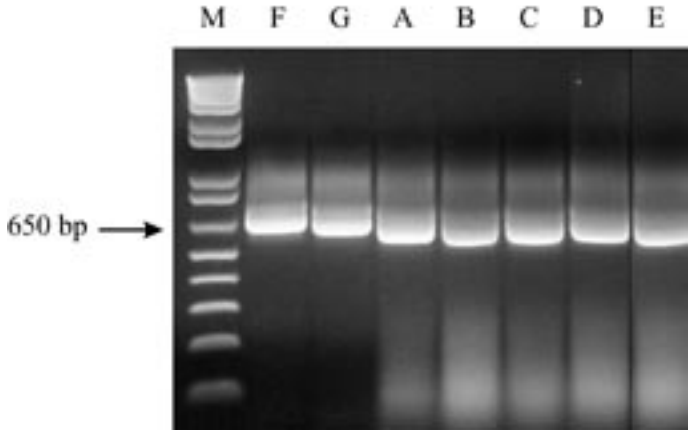

Figure 1. Electrophoresis of PCR products ORF6 and ORF7 genes of PRRSV on 2% agarose gel. The length of PCR products of the European genotype viruses was 647 bp and that of the North American genotype 670 bp. M – 1 Kb Plus DNA Ladder (Invitrogen), A – L-6791, B – Amervac-PRRS/A3, C – CAMP V-502, D – CAMP V-503, E – Porcilis PRRS, F – Ingelvac PRRS, G – VOS 2878

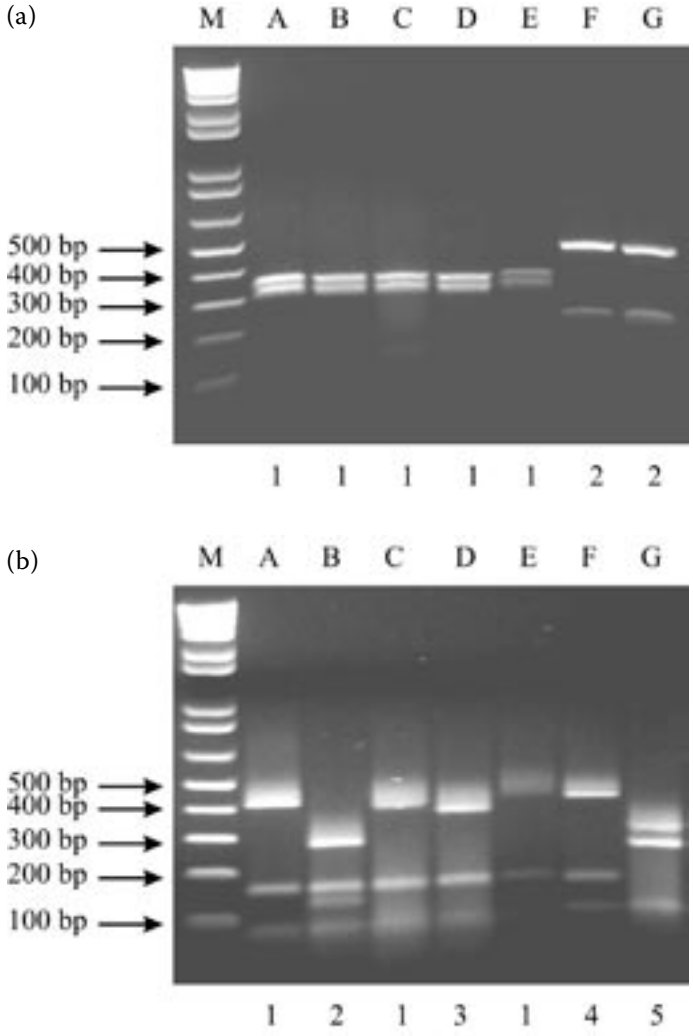

Figure 2. Types of RFLP patterns in PRRSV. PCR products (647 bp or 670 bp) containing ORF6 and ORF7 genes were digested with restriction enzymes Hae II and Alu I. Two types of RFLP patterns are shown for Hae II (a) and five different types for Alu I (b). Detection of digestion was carried out by electrophoresis on 2% agarose gel and stained with ethidium bromide. The length of fragments was determined based on detection of nucleotide sequences of PRRS viruses: Hae II – code 1 (339 bp, 308 bp), code 2 (454 bp, 216 bp); Alu I – code 1 (410 bp, 161 bp, 76 bp), code 2 (280 bp, 161 bp,130 bp, 76 bp), code 3 (378 bp, 161bp, 76 bp, 32 bp), code 4 (413 bp, 161 bp, 96 bp), code 5 (313 bp, 257 bp, 100 bp). Fragments less than 100 are poorly visible. M – 1 Kb Plus DNA Ladder (Invitrogen), A – L-6791, B – Amervac-PRRS/A3, C – CAMP V-502, D – CAMP V-503, E – Porcilis PRRS, F – Ingelvac PRRS, G – VOS 2878, 1–5 – RFLP codes

Ingelvac PRRS MLV and isolate VOS 2878/96 showed identity in the length of the restriction fragments (545 bp, 125 bp). The enzyme BsaJ I allowed differentiation of European and North American genotypes of PRRSV similarly to restriction digestion with the enzyme Hae II.

Combination of restriction enzymes Hae II, Alu I and BsaJ I yielded digestion patterns with five different RFLP patterns from twelve PRRS viruses under testing (Table 1). RFLP code 1-1-1 was the most frequent digestion pattern among the European type viruses and corresponded with all field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), isolate CAMP V-502, and vaccine strain Porcilis PRRS. Moreover, homogeneity could be seen in restriction patterns of the enzymes Hae II and BsaJ I in North American vaccine strain Ingelvac PRRS and isolate VOS 2878 (code 2-x-4).

DISCUSSION

Live attenuated vaccines based on the North American genotype Ingelvac PRRS (Boehring Ingelheim Vetmedica) and the European genotype Porcilis PRRS (Intervet) and Amervac-PRRS/A3 (Hipra) registered in the Czech Republic are commercially available for preventive vaccination of pigs against PRRS. Unlike inactivated vaccines, live attenuated vaccines have the advantage of more rapid induction of protective immunity, which makes the shedding period of the challenge virus shorter (Snijder and Meulenberg, 1998). The effectiveness of MLVs can be dependent on the vaccine strain. A considerable variability exists concerning the origin of the vaccine strains and phylogenetic differences between the European and North American strains, which can account for different efficacy. A limited efficacy of the North American

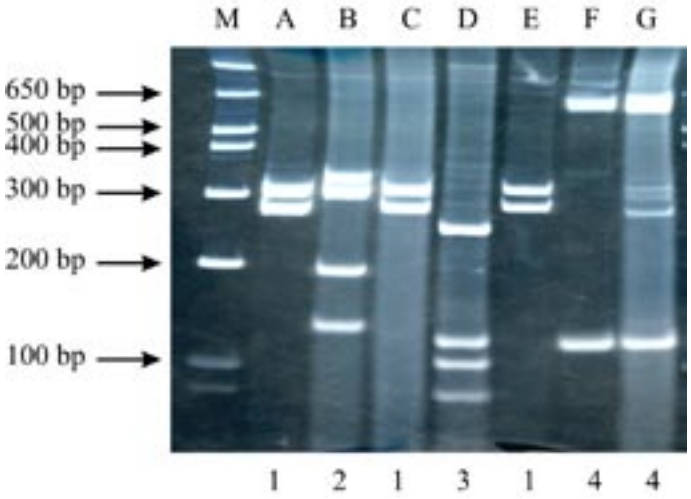

Figure 3. Types of RFLP patterns in PRRSV. PCR products (647 bp or 670 bp) containing ORF6 and ORF7 genes were digested with restriction enzyme BsaJ I. Four different types of RFLP patterns are shown for BsaJ I. Restriction digestion was detected by electrophoresis on 12.5% polyacrylamide gel and stained with silver nitrate. The length of fragments was determined base on detection of nucleotide sequences of PRRS viruses: BsaJ I – code 1 (315 bp, 283 bp, 49 bp), code 2 (330 bp, 315 bp, 190 bp, 140 bp), code 3 (247 bp, 125 bp, 106 bp, 84 bp, 49 bp, 36 bp), code 4 (545 bp, 125 bp). Fragments less than 100 are poorly visible. M – 1 Kb Plus DNA Ladder (Invitrogen), A – L-6791, B – Amervac-PRRS/A3, C – CAMP V-502, D – CAMP V-503, E – Porcilis PRRS, F – Ingelvac PRRS, G – VOS 2878, 1–5 – RFLP codes

genotype MLVs compared to the European types of field isolates is well known and this phenomenon has already been described (van Woensel et al., 1998; Labarque et al., 2003; Stadejek et al., 2005). High genetic variability among PRRSV isolates of the European genotype and the reference strain Lelystad contributed to explanation why the vaccine virus achieves higher efficacy at pig protection against homologous strains (Labarque et al., 2004). The results of current serological methods can only show whether particular animals or the whole herds came into contact with PRRSV but cannot distinguish vaccinated animals from those infected with a field strain. Live attenuated vaccine strains and field strains can persist in the vaccinated animals for several months. A test that is able to differentiate between infections caused by field strains and vaccine strains has been developed by Wesley et al. (1998). This procedure has been called restriction fragment length polymorphism (RFLP) assay. Wesley et al. (1998) used RFLP for differentiation of the North American vaccine strain RespPRRS from field strains of North American genotype.

Distinguishing between North American and European isolates as well as between field isolates and vaccination strains can be carried out by RFLP for PCR-amplified product containing ORF6 and ORF7 genes (Gagnon and Dea, 1998). As far as the restriction enzymes are concerned, BsaJ I and Alu I were regarded highly suitable for differentiation of the vaccine strain ATCC VR-2332 from field isolates.

In the submitted study, we monitored the range of genetic variability of ORF6 and ORF7 genes in MLVs, PRRSV isolates, and field viruses, obtained in the Czech Republic and in Slovak Republic, using RFLP method. RFLP analysis of twelve PRRS viruses resulted in 2, 4, and 5 different digestion patterns for the restriction enzymes Hae II, Alu I and BsaJ I. The selected set of restriction enzymes allowed a reliable differentiation of the European type and North American type viruses. The same digestion pattern (code 1) was described in all Czech field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), local field isolate CAMP V-502, and the vaccine strain Porcilis PRRS for both restriction enzymes Alu I and BsaJ I. The North American vaccine strain Ingelvac PRRS and the isolate VOS 2878 showed different RFLP pattern for the enzyme Alu I (code 4, 5) and BsaJ I (code 4) unlike of a cluster of viruses of the European genotype. A combination of restriction enzymes Hae II, Alu I and BsaJ I yielded five different RFLP patterns in twelve of the tested PRRS viruses (Table 1). RFLP code 1-1-1 was identical in all field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791), isolate CAPM V-502, and the vaccine strain Porcilis PRRS. This conformity is suggestive of higher antigenic similarity of the vaccine strain of the European genotype Porcilis PRRS with the Czech isolate CAMP V-502 and field viruses (L-588, L-1606, L-2053, L-3305, L-6558, L-6791) compared to other PRRS vaccines. Besides, homogeneity in restriction patterns of Hae II and BsaJ I in the North American vaccine strain Ingelvac PRRS and isolate VOS 2878 (code 2–4) could be seen.

The basis for a suitable use of RFLP analysis is the fact that RFLP pattern of PRRSV is relative-

ly stable during the infection although potential changes can occur (Wesley et al., 1998; Wesley et al., 1999). Finding of new RFLP pattern during a certain period of time has been described by Cai et al. (2002). Moreover, high degree of genetic variability was confirmed among field isolates of PRRSV with the same digestion RFLP patterns and instability of RFLP patterns PRRSV during *in vitro* replication (Cha et al., 2004).

The obtained results indicate that determination of RFLP profiles in a genome site containing ORF6 and ORF7 genes can be a valuable tool for differentiation of PRRSV isolates and vaccine strains. The fact that a genome site corresponding to ORF6 and ORF7 genes of PRRSV appears as a region with higher sequence homology than ORFs 3–5 makes this region valuable for identification of PRRSV strains (Gagnon and Dea, 1998; Oleksiewicz et al., 1998). Another reason is that this region is present not only in genomic RNA but also in all subgenomic RNA molecules and replication intermediate product with negative polarity. Besides, a certain correlation may occur among the antigenic variants determined at reaction to anti-M and anti-N MAbs, and RFLP profiles obtained by different restriction enzymes indicate that some nt substitutions can result in changes having impact on epitopes which are recognised by MAbs (Gagnon and Dea, 1998). Therefore the use of primers selected from this region allows the highest possible sensitivity of reaction. Several studies have been devoted to RFLP analysis from this gene region (Gagnon and Dea, 1998; Umthun and Mengeling, 1999; Cai et al., 2002). RFLP analyses from part of PRRSV genome of ORF5 gene which codes for envelope glycoprotein E and as an antigenic agent is connected with virus neutralization suggest a possible high number of deviations in RFLP profiles (Cheon and Chae, 2000; Itou et al., 2001; Indik and Valicek, 2002).

The results of PCR-based RFLP analysis of PRRSV isolates and MLVs can be used in epidemiological studies of PRRS in pig herds. Field strains of PRRSV can be compared with MLVs registered in the Czech Republic or other countries, and the results can be used in selecting a vaccine with maximum genetic similarity to the circulating field PRRS viruses.

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