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Identification of a genetically diverse sequence of porcine reproductive and respiratory syndrome virus in Slovenia and the impact on the sensitivity of four molecular tests☆

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A total 91 serum samples and 51 pig tissue samples were collected between October 2009 and June 2010 from 30 herds, where a clinical picture of infection or/and porcine reproductive and respiratory syndrome (PRRS) antibody-positive pigs were detected. Of the 142 samples tested, 65 (45.8%) were identified as porcine reproductive and respiratory syndrome virus (PRRSV) positive by a one-step reverse transcription and polymerase chain reaction (RT-PCR). The sequencing results of 258 nucleotides in ORF7 from 30 herds with PRRSV-positive samples revealed the circulation of six genetically different strains of PRRSV, all belonging to the Subtype 1 (Type I). Twenty-three (76.6%) of the thirty positive herds were infected with a genetically identical cluster, with 98.9–100% nucleotide identity between the herds, representing the detection of a new strain of PRRSV in Europe, not published previously. From these 23 herds, positive PRRSV samples were detected with gel-based RT-PCR, but all gave false-negative results with two commercial real-time kits. When using a third commercial real-time kit, 28 (93.3%) of 30 positive samples in gel-based RT-PCR were detected as the Type I, confirming that the sensitivity of this real-time kit is much greater than the sensitivity of the previous two. The influence of new genetic variants of PRRSV circulating in Slovenia on molecular diagnosis and the control of the infection is discussed.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped, positive, single-stranded RNA virus of the Arteriviridae family (Cavanagh, 1997). The genome consists of nine open reading frames (ORFs) which code the viral replicase (ORF1a and 1b), four membrane-associated glycoproteins (ORF2a and 5), two unglycosylated proteins (ORF2b and 6) and the nucleocapsid (N) protein (ORF7) (Meng et al., 1994). The most immunogenic protein in the virion is the 15 kDa N protein and it is an important target region for viral detection by RT-PCR and serological detection by ELISA. Kang et al. (2004) noted that the phylogenetic tree derived from ORF7 resembles most closely the tree derived from full-length genomes of PRRSV, thus this region might be used as a useful marker for revealing the genetic relationships of PRRSV. The causative agent of PRRS was first described in Europe, where it was named Lelystad virus (Wensvoort et al., 1991), and in the United States, with the detection of the reference strain VR-2332 (Benfield et al., 1992). The European isolates were first thought to form a very homogeneous "Lelystad-like" group of PRRSV (Drew et al., 1997); however, this was challenged by reports of diverse genotype PRRS strains with 72.2–90% homology from many countries including Denmark, Italy, Czech Republic, Poland, Spain, Germany, Netherlands, Thailand and Belorussia (Indik et al., 2000; Oleksiewicz et al., 2000; Stadejek et al., 2002; Mateu et al., 2003; Thanawongnuwech et al., 2004; Pesch et al., 2005; Stadejek et al., 2006).

PRRSV can be diagnosed by virus isolation, detection of antigens or nucleic acids in infected tissues and body fluids. However, there are several potential problems with the diagnosis of PRRS because of the wide variability of the strains. The molecular-based reverse transcription polymerase chain reaction (RT-PCR) method is one of the techniques used most commonly for PRRSV detection in infected tissues, serum and semen. Recently, real-time RT-PCR technology using primers and TaqMan probes has been used successfully to detect the genotype and the quantity of PRRSV RNA (Egli et al., 2001; Chung et al., 2005). These assays are sensitive and achieve high specificity through the gene-specific primers and probes. The main disadvantages are the design and the validation of specific functional probes to ensure the diagnostic sensitivity and

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specificity of the assay and the avoidance of false-negative results due to variability within the probe-binding site (Hughes et al., 2004). The SYBR green-based real-time PCR assay and PriProET assay followed by melting curve analysis for the detection and genotyping of PRRSV was used as an alternative to avoid false-negative results (Martinez et al., 2008; Balka et al., 2010).

At the end of 2009, the Slovenian pig population consisted of 21,952 herds with 353.170 pigs. The majority of pigs were located in the eastern part of the country. More than half of the pig populations are kept on large units with 800–5500 sows per unit, but other pig herds are rather small with 10–1000 pigs per herd. A serological survey on swine sera during 1999–2004 in Slovenia had demonstrated that the herds were free of PRRSV (Valenčak, 2004). Only few years later the detected percentage of sero-positive herds was 44.8%, as indicated by the data of a study on antibody prevalence in 194 herds in 2010 (Toplak et al., 2010).

The objective of this study was to determine PRRSV from clinical samples collected during 2009–2010 using conventional RT-PCR and to validate the suitability of three different commercial real-time PCR kits for the detection of circulating PRRSV field strains. Direct sequencing of the amplified part of ORF7 from positive samples collected from 30 selected herds was performed to determine the genetic heterogeneity of the current PRRSV in Slovenia.

2. Materials and methods

2.1. Field samples

In total, 142 samples (91 sera and 51 dead pig tissue samples) were collected from 30 herds during October 2009 and June 2010. Samples were collected in pig herds due to either acute respiratory disease in growing pigs or late-term abortions in sows. Fifty-one tissue samples (lungs, spleen and lymph nodes) were collected from individual dead pigs and sent to the pathology unit for necropsy. One g of tissue samples from individual pigs was homogenized as a pool in RPMI-1640 (Gibco, Paisley, UK) with a homogenizer (IUL instruments, Königswinter, Germany). After homogenization, the suspensions of samples were centrifuged for 15 min at 3000 × *g*. The tissue supernatant was recovered and used for the extraction of total RNA.

2.2. Detection of PRRSV with gel-based RT-PCR using primers for the detection of Type I and Type II strains

Total RNA was extracted from 140 μl of tissue supernatant or serum samples using the QIAamp® viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One-step RT-PCR (One-Step RT-PCR Kit, Qiagen, Hilden, Germany) was performed using degenerated primer sequences based on the open reading frame 7 (ORF7), with the forward primer P1 5′-CCA GCC AGT CAA TCA RCT GTG-3′ and the reverse primer P2 5′-GCG AAT CAG GCG CAC WGT ATG-3′, which detect both Type I and Type II of PRRSV strains (Donadeu et al., 1999). The PRRS strain VR-2332 (Type II) and the Lelystad viruses (Type I) were used as the positive controls. Reaction mixtures without RNA served as negative controls. The reaction was performed in a total volume of 25 μl as follows: 15 μl of nuclease free water, 5 μl of 5× PCR buffer, 1 μl of dNTP mix (containing 10 mM of each dNTP), 0.5 μl of the stock solution with 20 μM of each primer, 1 μl of the one-step RT-PCR enzyme mix and 2 μl of the RNA template. The RT-PCR program included a reverse transcription stage at 50 °C for 30 min, followed by an initial PCR activation step at 95 °C for 15 min. This was followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The reaction was performed using a T1 thermal cycler (Biometra, Horsham, USA) and the RT-PCR products were visualized in 1.8% agarose gel with 0.5 μg/ml ethidium bromide and subsequent visualization under a UV light. The size of the PCR product was compared to the GeneRuler™ 100 bp DNA Ladder (Fermentas, St. Leon-Rot, Germany), and PCR products of about 300 bp were interpreted as positive according to the expected DNA fragment and negative if no specific product was visible.

2.3. Sequencing and phylogenetic analysis

The PRRSV from 30 herds were directly sequenced from 30 RT-PCR products in both directions using the Macrogen sequencing service (Macrogen, Geumchen-gu, South Korea) and the RT-PCR amplification primers to confirm the specificity of the RT-PCR products. For each sample, 258 nucleotide long sequences were aligned with the published data using BLAST (available at http://www.ncbi.nlm.nih.gov/) at the National Centre for Biotechnology Information (NCBI). Multiple sequence alignment was carried out using the sequence analysis software Lasergene® (DNASTAR, Madison, WI, USA) and a phylogentic tree was generated using PHYLIP and the Clustal W alignment algorithm. In order to assess the statistical reliability of the dendrograms, bootstrapping values were calculated (random numbers seed: 123; 1000 replicates) (Felsenstein, 1989).

2.4. Detection of PRRSV with three commercial real-time PCR kits

In order to determine the diagnostic sensitivity of three commercial real-time kits, TaqVet® PRRS Triplex PRRS-Genotyping (LSI, Lissieu, France), ADIAVET® PRRS EU/NA (Adiagene, Saint-Brieuc, France) and NextGen Real-Time RT-PCR Target Specific Reagents for the Detection & Differentiation of North American & European PRRSV viral RNA (Tetracore®, Rockville, USA), named in following sentences as kit A, B and C were tested. The same batch of aliquots of 30 extracted RNA as for gel-based RT-PCR method (Donadeu et al., 1999) was used for the detection of PRRSV with each kit and individual real-time test was performed and interpreted according to the manufacturer's instructions. According to the producer's specifications, the three real-time kits allow the detection of a sequence of the ORF7 in the viral genome of PRRSV in the same well with the simultaneous detection and differentiation between Type I and Type II strains and the detection of internal positive control using specific primers and specific dye-labeled probes.

3. Results

3.1. Detection of PRRSV with gel-based RT-PCR

Of the 142 tested samples, 65 (45.8%) were identified as PRRSV positive (detection of RT-PCR products of about 300 bp) by gel-based RT-PCR. PRRSV positive samples were detected in 30 different pig herds, each representing a new location. The majority of the PRRSV-positive samples were detected in the eastern part of Slovenia (Štajerska and Pomurje regions), where the highest density of pig producing farms is located (Table 1).

3.2. Sequencing results of the strains detected in 30 different herds

The sequencing results of the partial nucleocapsid protein gene (258 nucleotides of ORF7, genome position 14.673–14.927 in the Lelystad virus, GenBank accession number M96262) from the 30 positive representative samples detected in 30 herds revealed the circulation of six distantly related PRRSV strains (Fig. 1). The detected PRRSV shared an 89.1–96.1% nucleotide identity with the Lelystad virus (Type I), and a 62.4–65.1% nucleotide identity with

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Table 1
Origins of the PRRSV strains detected in this study and the results of the PRRSV detection by four molecular tests (gel-based RT-PCR and three commercial real-time RT-PCR kits: kit A, kit B and kit C).

Herd no.	Isolate	Municipality name	Accession no.	RT-PCR	Kit A Type I	Kit B Type I	Kit C Type I	Kit C Type II
1	SI-Stub5/2009	Branik	HQ213910	+	33.17	29.70	29.85	29.23
2	SI-Ziz3/2009	Beltinci	–	+	Undet.	Undet.	26.85	27.39
3	SI-Stra8t/2010	Videm ob Ščavnici	HQ213911	+	Undet.	Undet.	30.44	31.88
4	SI-Nem26/2010	Beltinci	HQ213912	+	Undet.	Undet.	26.08	27.05
5	SI-14/2010	Beltinci	HQ213913	+	Undet.	Undet.	30.23	32.05
6	SI-2581/2009	Apače	HQ213914	+	Undet.	Undet.	14.45	15.27
7	SI-2PI/2009	unknown	HQ213915	+	Undet.	35.64	Undet.	31.12
8	SI-5PI/2009	unknown	HQ213916	+	Undet.	Undet.	39.94	Undet.
9	SI-9PI/2009	unknown	HQ213917	+	Undet.	Undet.	39.00	37.70
10	SI-586/2010	Tišina	HQ213918	+	Undet.	Undet.	29.84	32.00
11	SI-1895/2010	Gornji Petrovci	–	+	Undet.	Undet.	27.36	27.22
12	SI-1903/2010	Turnišče	HQ213919	+	Undet.	Undet.	Undet.	Undet.
13	SI-1904/2010	Murska Sobota	–	+	Undet.	Undet.	32.40	Undet.
14	SI-2050/2010	Šmarješke Toplice	–	+	Undet.	Undet.	30.58	37.33
15	SI-2080/2010	Preddvor	HQ213920	+	27.23	27.37	27.61	31.00
16	SI-2108/2010	Gornja Radgona	HQ213921	+	30.08	24.18	26.45	Undet.
17	SI-2111/2010	Ljutomer	HQ213922	+	28.84	27.94	28.43	28.81
18	SI-2113/2010	Videm ob Ščavnici	HQ213923	+	35.06	40.05	25.20	29.42
19	SI-2114/2010	Videm ob Ščavnici	–	+	Undet.	Undet.	33.01	Undet.
20	SI-2115/2010	Križevci pri Ljutomeru	–	+	Undet.	Undet.	37.00	Undet.
21	SI-2106/2010	Puconci	HQ213924	+	42.65	35.04	32.67	Undet.
22	SI-2107/2010	Rogašovci	HQ213925	+	Undet.	32.98	26.35	Undet.
23	SI-1855/2010	Šmarje pri jelšah	HQ213926	+	Undet.	Undet.	22.80	21.92
24	SI-964/2010	Ljutomer	HQ213927	+	Undet.	Undet.	32.28	Undet.
25	SI-965/2010	Tišina	HQ213928	+	Undet.	Undet.	33.47	Undet.
26	SI-1262/2010	Križevci pri Ljutomeru	HQ213929	+	Undet.	Undet.	35.57	Undet.
27	SI-1265/2010	Beltinci	HQ213930	+	Undet.	Undet.	28.46	Undet.
28	SI-0806/2010	Murska Sobota	HQ213931	+	Undet.	Undet.	28.28	30.29
29	SI-3945/2010	Murska Sobota	HQ213932	+	Undet.	Undet.	31.83	Undet.
30	SI-4142/2010	Dol pri Ljubljani	HQ213933	+	36.73	Undet.	19.38	22.67
% Of positive				30/30 100%	6/30 20.0%	8/30 26.6%	28/30 93.3%	16/30 53.3%
Sensitivity				100%	55.5%	57.6%	93.7%	–

Interpretation for gel-based RT-PCR; +: PRRSV positive in gel-based RT-PCR. Interpretations for real-time RT-PCR kits A (Type I only), B (Type I only), C; cycle threshold value (ct) <45: positive result in real-time RT-PCR, Undetected (Undet.): negative result, when ct was >45.

VR-2332 (Type II). On the amino acid level, the detected PRRSV revealed an 88.4–98.8% identity with the Lelystad virus. In 76.7% of the positive Slovenian herds from this study, genetically closely related strains were identified (representative strain SI-4142/2010) with a 97.7–100% nucleotide and amino acid identity. This group of viruses, detected in 23 infected pig herds, represented the detection of a new group of PRRSV in Europe, with only a 92% nucleotide identity to the most closely related sequence of the PRRSV strain 340-1 (AY035959) from the Netherlands. The other seven herds (23.3%) were infected by five genetically distant PRRSV with an 88.8–99.6% nucleotide identity to each other and with an 85.7–96.5% identity to the published strains from Europe. The phylogenetic tree constructed from 258 nt sequences of ORF7 from this study and the representative PRRSV strains for Type I; Subtypes 1, 2, 3 and 4 (Stadejek et al., 2006) revealed a similar topology to the previously published strains in ORF5, confirming that all of the detected strains in this study were clustered into Subtype 1 (Fig. 1).

3.3. Detection and sensitivity of the three commercial real-time kits A, B and C

After the first laboratory observation of false-negative results obtained with PRRSV suspected field samples, when using the commercial real-time kit A, gel-based RT-PCR was implemented as the first method after successful internal laboratory validation for screening all suspected samples. Before this study, there was no available data on the genetic variability of circulating field strains of PRRSV in Slovenia. A one-step gel-based RT-PCR assay for detecting Type I and Type II of PRRSV strains and a rapid-direct sequencing protocol for typing PRRSV was used as "gold standard" in this study. The sequencing results of data from the highly conserved region of ORF7 revealed a high genetic diversity of our PRRSV strains, and the sensitivity of the three commercially available real-time PCR kits for the detection of field PRRSV strains was evaluated. With the real-time kit A only 6 (20.0%) out of 30 PRRSV positive samples (Subtype 1) were detected; all of the samples were negative for the Type II strains. A very similar low sensitivity (57.6%) was obtained with the second commercial real-time kit B, with the detection 8 (26.6%) samples as positive out of the 30 PRRSV positive field samples by RT-PCR. Both commercial real-time kits A and B failed to detect almost all of the samples which were clustered into the group of PRRSV strains detected in 23 herds in this study (Fig. 1). But all 6 and 8 samples that were found to be positive by these two real-time PCR kits were genotyped as Type I of PRRSV (Table 1). Twenty-eight (93.3%) of the previously determined positive samples (by RT-PCR) were positive with the kit C, using Type I probes (Cy3 dye), which showed the best sensitivity (93.7%) when the results of the three tested commercial real-time kits were compared to detected PRRSV by RT-PCR (Table 1). Only two samples (SI-2PI/2009 and SI-1903/2010) were not correctly detected with the real-time kit C (Table 1). The sample with name SI-2PI/2009 was found to be positive for the Type II (cycle threshold (ct) value 31.12), and negative (ct value >45) with the Type I probe, but our 258 nt sequencing results confirmed that this strain belonged to the Type I of PRRSV (Fig. 1). According to the specifications of the producer of the real-time kit C, the Type I of PRRSV is present in the sample if positive ct value with Type I probes and positive or negative ct values with the Type II probes was detected. The low sensitivity of the two commercially available real-time kits A and B was evident for most of the currently circulating PRRSV field strains, and is the result of

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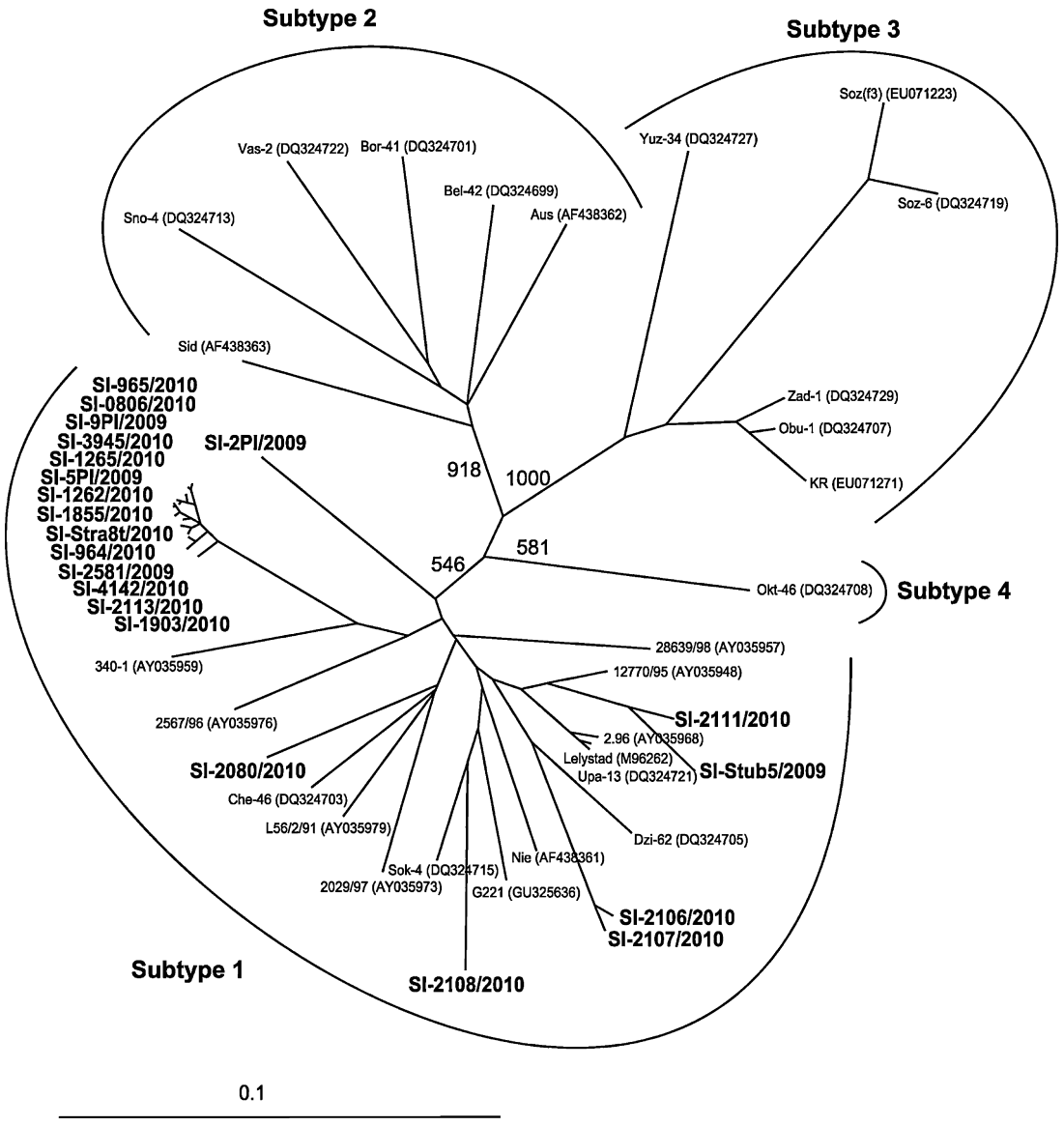

Fig. 1. The phylogenetic tree was constructed using 21 unique partial ORF7 nucleotide sequences (258 nt) from this study (bold), including 27 representative sequences (258 nt) of Type I; Subtypes 1, 2, 3 and 4 isolates.
Classification of subtypes according to Stadejek et al. (2006).

the high prevalence of the new genetic variant of PRRSV, which was detected in 23 herds; however, the majority of the samples from the other five genetic variants of PRRSV were correctly detected by the three real-time kits A, B, C. Two positive samples (SI-2PI/2009 and SI-2107/2010) were negative by the real-time kit A, representing the lower sensitivity (55.5%) of this kit in comparison to the real-time kits B (57.6%) and C (93.7%). Sample SI-4142/2010 was found to be positive with the real-time kit A although it belongs to the new group of PRRSV, but the ct value was much higher (ct = 36.73), compared to ct value of 19.38 obtained by the kit C (Table 1, Fig. 1).

4. Discussion

The aim of this study was to analyse the genetic diversity of 30 field strains detected in PRRSV infected herds in Slovenia and to evaluate their impact on diagnostic sensitivity of three available commercial real-time kits. After the recognition of false negative results with commercial real-time kit A, the sensitivity of three commercially available real-time kits A, B and C was assessed with the 30 PRRSV herd positive samples and the results were compared to the gel-based RT-PCR. Because PRRSV was new infection introduced into the country at the beginning of 2005, no preventive measures were put in place against PRRSV, before high economic losses were observed for the first time in infected herds and at the beginning many pig owners were not aware of the consequences of the PRRSV introduction into the herds. The herds included in this study had reported clinical signs of the PRRS disease, representing with, acute respiratory disease and 20–50% mortality among growing pigs, reproductive and/or respiratory disorders in sows and after infection of pigs the majority of infected herds were using a vaccination strategy to prevent economic losses. A recent commercially available real-time kit A, was used in laboratory between 2009 and 2010 to detect PRRSV in suspected samples. According to the real-time kit producer's data, this kit was validated on field samples and can detect and differentiated both Type I and Type II PRRSV simultaneously with two specific probes. Because kit A allows the pooling of samples and is using an internal control, this kit has been

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used for PRRSV diagnosis in Slovenia. However, after 4 months of testing on PRRSV suspected samples from different herds, false-negative results were recognized, probably due to the unrecognized heterogeneity of field strains in large number of PRRSV infected herds.

In this study, the circulating PRRSV field strains in 30 infected herds with the published primers described by Donadeu et al. (1999) were detected. Of the 142 samples tested by gel-based RT-PCR, 45.8% were positive, confirming that PRRSV was detected in a high percentage of the clinically suspected herds. The sequencing results of the partial N protein (258 nt of ORF7) of PRRSV from the 30 positive herds revealed the circulation of six PRRSV strains that are related distantly to the Lelystad virus. These data suggest that many introductions of PRRSV into Slovenia occurred between 2004 and 2010 from many unknown locations, probably as a result of the importation of live PRRSV infected pigs.

The detection of new genetic groups of PRRSV in this study enlarged the known diversity of detected strains. The limited number of field strains deposited in GenBank for the ORF7, revealed that the majority of viral introductions in Slovenian were unable to connect with published strains, what is similar to observation of other investigations (Indik et al., 2005; Pesente et al., 2006; Balka et al., 2008; Greiser-Wilke et al., 2010), but in future the detection of similar strains can be expected in other countries. Recently, when studying isolates from eastern European countries (Belarus, Lithuania and Poland), Stadejek et al. (2006) divided the PRRSV Type I into four genetic subtypes comprising Subtypes 1, 2, 3 and 4. Even though the phylogenetic tree was constructed using only 258 nt sequences and compared with representative PRRSV strains of Subtypes 1, 2, 3 and 4 (Stadejek et al., 2006) of ORF7, it revealed a greater similarity of clustering of the viruses found in complete ORF5 and ORF7 trees, confirming that all of the detected strains from this study were clustered into Subtype 1 (Fig. 1). The three PRRSV positive samples from this study were sequenced in complete region of ORF5 (606 nt) (data not shown) and the closely related sequences in GenBank using BLAST tool were determined. PRRSVs detected most commonly in Slovenia, with representative strain SI-4142/2010, revealed 92–95% nucleotide identity with the 27 sequences of PRRSVs from Italy (IT60, IT35, IT14, IT37, IT32, etc.) published in 2006 (Pesente et al., 2006). PRRSV named SI-2080/2010 revealed 93–94% nucleotide identity to the Spanish PRRSV (Spain 5/1992 – DQ345732, CReSA-38 – EF429113, Cresa3267 – JF276435). PRRSV named SI-2PI/2009 revealed 89% nucleotide identity in the ORF5 to the PRRSV strain O8V194 – GU737265 published in Belgium. This observation supports the finding that similar strains of PRRSV as detected in Slovenia were published previously in Europe, but not related closely.

This study also confirmed that routine direct sequencing of PCR products can be an important tool for diagnostic laboratories and support the value of rapid recognition of new PRRSV field strains to explain possible unexpected low sensitivity of some commercial real-time kits or other RT-PCR methods.

The routine diagnostic gel-based and real-time PCR protocols in published studies were often modified and adjusted to fulfil the requirements in the field. The first false-negative results with the commercial real-time kit A were suspected after 4 months of the parallel testing of herds for PRRSV and antibodies with ELISA (HerdChek PRRS X3, IDEXX, Westbrook, ME, USA). After the sequencing of PRRSV and the detection of a new genetic group in 23 out of 30 herds, the low sensitivity with 55.5% and 57.6% detected positive samples were found for the real-time kits A and B, respectively, and this result almost completely matched the number of detected PRRSV strains clustering into new detected group of PRRSV from this study (Fig. 1). Although both real-time kits were detected the other five genetic variants in our PRRSV positive samples, they did not detect the majority of PRRSV positive herds, because these herds were all infected with new genetic variant of PRRSV. The third commercially available real-time kit C was validated on the same 30 selected PRRSV positive field samples with the detected sensitivity of 93.7% (Table 1). Twenty-eight positive samples were correctly detected as positive Type I of PRRSV, but sample SI-2PI/2009 was detected as positive for the Type II, although in the sequencing results, this PRRSV sample was clustered within Subtype 1 (Fig. 1). The second sample, SI-1903/2010, was found to be negative, although a 100% identical sequence was observed in the sequenced region (258 nt), as in sample SI-1904/2010, which was recognized with ct value 32.40. Because there were no available data about the positions and sequences of the primers and probes in ORF7 (or other regions of viral genome) for any of the three commercial real-time kits, it can be concluded that data obtained for the selected 30 positive samples is the result of differences in primer and probe matching with the positive field samples. Although the results of many experiments confirmed specificity for each published real-time protocol with a large number of tested PRRSV samples (Egli et al., 2001; Balka et al., 2010), the results with a large number of false-negative results confirmed the importance of continuously routine genotyping of PRRSV field strains. Regular revalidation of each test for the detection of PRRSV is important and should be considered as high priority before using RT-PCR methods as a diagnostic tool in the diagnostic laboratory.

The results of this study demonstrated that infection with uncommon PRRSV strains occurred in 76.7% of 30 PRRSV-positive pig herds, and that the majority of pigs infected with this new strain could not be detected by two commercial real-time kits. These results should be considered for the diagnosis and control of PRRSV in neighbouring countries. A new approach that follows up the current position with sequencing of all PRRSV field strains which are detected becomes an important tool for the laboratory to improve the diagnosis and control of this infection.

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