Jūratė Buitkuvienė

EPIDEMIOLOGICAL INVESTIGATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) IN LITHUANIAN WILD BOARS AND PIGS POPULATION

Summary of Doctoral Dissertation
Agricultural Sciences, Veterinary Medicine (02A)

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Jūratė Buitkuvienė

KIAULIŲ REPRODUKCIJOS IR KVĖPAVIMO SINDROMO VIRUSO (KRKSV) EPIDEMIOLOGINIAI TYRIMAI LIETUVOS ŠERNŲ IR KIAULIŲ POPULIACIJOSE

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INTRODUCTION

The porcine reproductive and respiratory syndrome virus (PRRSV) containing 15 kb of RNA and belonging to the family *Arteriviridae*, which emerged practically during the same period on all the continents more than 25 years ago, is widespread in the pig populations of many countries. It causes reproductive failure in sows, and respiratory tract illness in other pigs and, in young pigs of different age, in particular. The disease was reported for the first time in the United States of America and Canada in 1987 (Loula, 1990; Keffäber, 1989), however, it was only in 1990 in Europe and in 1992 in the USA that the agent of the disease was isolated (Weensvoort et al., 1991; Collins et al., 1992). Wenhsvoort and his colleagues isolated the agent of the disease from the pigs infected with this disease and named it Lelystad virus. In the same year a similar virus was isolated by Louis Harris in the United States, which later, according to the national classification of viruses, was identified as the virus strain ATTCC VR-2332. These strains were the first to have been identified as the earlier unknown species of viruses and currently known as porcine reproductive and respiratory syndrome (PRRS). These discoveries helped divide PRRS into the following two genotypes that are generally recognised at the present time: the European genotype (PRRSV-1), and the North American genotype (PRRSV-2), i.e. the United States genotype (Snijder et al., 2004).

The first clinical symptoms of porcine reproductive and respiratory syndrome in Lithuania were first observed at the beginning of 1997, specific antibodies of PRRSV were determined (Janutėnaitė et al., 2000). High prevalence of PRRS, later characteristic enzooticity, as well as preliminary ELISA (enzyme-linked immuno sorbent assay) studies testified to the relevance of this disease in Lithuania.

Though the disease is highly detrimental and serious, PRRSV infection has not been investigated extensively in Lithuania. The seroprevalence of PRRSV has been episodically studied only several times on different pig-breeding farms (Stankevičienė et al., 2002; Stankevičienė et al., 2008). Moreover, molecular diagnostics has been introduced and PRRSV strains have been characterised (Stadejek et al., 2002; Stankevičius et al., 2003; Stankevičius et al., 2008) whose ORF5 nucleotide sequences were only 71.5 per cent identical with the strains prevalent in Western Europe (Stadejek et al., 2002; Stankevičius et al., 2003a). These sequences, as compared with any other PRRSV nucleotide sequences analysed earlier in Europe, turned out to be very different. The latest results of phylogenetic investigations revealed that similarly to the case of the Lithuanian populations, especially different PRRSV strains of the European genotype were prevalent in the pig populations in other countries of Eastern Europe. On the basis of the analysis of nucleotide sequences in the regions of ORF5 and ORF7 strains, the PRRSV strains prevalent in Europe can be divided into three clearly different subtypes: the Lelystad strain, a similar Western European PRRSV group (the 1st subtype) and two Eastern European PRRSV groups (the second subtype and the third subtype) (Stadejek et al., 2006, 2007, 2008).

Detailed investigations into the PRRSV strains have been carried out in Lithuania and Latvia (Stankevičius et al., 2008). The characterisation of PRRSV in ORF5 and ORF7 regions showed that PRRSV strains of the second subtype were prevalent in the pig populations in Lithuania, whereas the strain of the first subtype characteristic of the pig population of Western Europe has been determined on one farm only. The new, earlier unknown fourth subtype of PRRSV has been determined on the pig-breeding farms in Latvia. Its strains formed a separate group in the phylogenetic trees of ORF5 and ORF7 nucleotide and amino acid sequences to which only these Latvian PRRSV strains and the only Belarusian PRRSV strain that was determined earlier belonged.

During the past 10 years in a row, entire pig-breeding technology and conditions have changed in Lithuania, live attenuated vaccines have been used for prophylaxis of the prevention of PRRSV infection, not only the breeds of pigs raised on the farms have changed but also strict measures of biological safety have been started to be implemented. Seeking to elucidate how these changes influenced the prevalence of PRRSV on pig-breeding farms in Lithuania, and what situation of PRRSV infection has formed on the present-day pig-breeding farms in Lithuania, the necessity arose to carry out new investigations and to evaluate the distribution of PRRSV-specific antibodies in different groups of pigs, on the farms and in the regions.

At the present time methods of determining ELISA are widely used both in Lithuania, in the European Union and in the world when carrying out PRRSV infection monitoring programmes. The early and timely determination of PRRSV-specific antibodies enables the PRRSV infection situation to be effectively controlled in the negative or low risk swine herds, however, it is important to know exactly whether commercial ELISA kits are sufficiently accurate, sensitive and reliable to determined the antibodies of PRRSV strains of both genotypes. Also, it is important to make clear how the research results can be influenced by the parameters of homogeneity and stability of the samples, and whether ELISA kits intended for pigs are sufficiently accurate in establishing PRRSV-specific antibodies in blood sera of wild boars. Work with the samples of blood sera of both pigs and wild boars carried out encompassed all these practical questions of significance to the serological assay that were related to PRRSV antibody
studies. Also, drawing attention to the fact that wild boars, which according to a scientific classification, belong to the same type, class, order as pigs do, are considered to be one of the agents of many dangerous infectious diseases, which can infect domestic pigs, animals of other species and people (Ruiz-Fons et al., 2008), they can be a source of swine infectious diseases (Laddomada, 2000; Al Dahouk et al., 2005), it was decided to carry out PRRSV investigations into the population of wild boars for the first time in Lithuania. The fact that during the recent years wild boards have been treated as the object of the hunt in Lithuania, their population has increased considerably. According to 2010 accounting made by the Lithuanian Department of Statistics, the number of wild boars that live in freedom in Lithuania totals 54,608, and in 2011 this figure stood at as many as 57,805 wild boars. It is important to mention the fact that as compared with 280 wild boars living in freedom that were registered Lithuania in 1934, their population has increased by as many as 200 times.

Interaction and exchange of the agents of the disease between wild boars and domesticated pigs are studied and described best in the cases of classical swine fever and viral infection of Aujeszky’s disease (Albina et al., 2000). Wild boars are considered to be the main source of these viruses. There are very little data about PRRSV infection in wild boars in scientific literature available, but it is known that this virus causes respiratory tract illnesses in young pigs, and reproductive disorders in sows.

Antibodies against PRRSV in wild boars were determined in several countries and in isolated cases only (Oslage et al., 1994; Saliki et al., 1998; Albina et al., 2000). Many other publications reported on negative results of the investigations into PRRSV-specific antibodies (Vicente et al., 2002; Ruiz-Fons et al., 2006; Zupančič et al., 2002; Vengust et al., 2006). At the present time it is thought that it is domestic pigs that infect wild boars with PRRS virus and not vice versa, however, there is no conclusive evidence regarding that, the data are insufficient and they are often contradictory. The data currently available fail to present any clear evidence about wild boars being a source of PRRSV (Ruiz-Fons et al., 2007; Meng et al., 2009). It is likely that domestic pigs could have become infected from the wild boards earlier when there were no strict biosafety requirements set to the pig-breeding farms. The present investigation was carried out seeking to evaluate the significance of PRRSV infection in the wild boar population in Lithuania.

The objective of the present study
To evaluate the epidemiological situation of PRRSV infection in the wild boar and pig populations in Lithuania by means of immunological and molecular methods.

Goals of the study
1. To carry out serological PRRSV studies on pig-breeding farms in the regions of Lithuania, pig herds or groups of different sizes.
2. To carry out serological PRRSV infection studies in the population of wild boars in Lithuania establishing the antibodies specific to this virus in different age groups of the animals.
3. To evaluate the reliability and accuracy of the enzyme-linked immunosorbent assay method by establishing specific antibodies in the samples of pig and wild boar blood serum and employing ELISA diagnostic kits made by different manufacturers and standard sera for this purpose.
4. To test oligonucleotide primers AT-nPGR of ORF5 and ORF7 region used in the diagnostics of porcine PRRSV when studying blood sera and lung tissue samples of wild boars, and to find out whether PRRSV circulates actively in the wild boar population in Lithuania.
5. To compare phylogenetically ORF5 nucleotide sequences of PRRSV established in the wild boar population with the reference PRRSV sequences of different subtypes of the European genotype prevalent in the pig population.
6. To characterise PRRS virus strains circulating in the wild boar population in Lithuania for the immune response in the important region encoding GP5 protein.

Scientific innovation and significance of work
1. The serological studies of PRRSV carried out on the pig-breeding farms in Lithuania showed that the changed pig-breeding conditions, technologies and the adopted biosafety measures, as well as new groups of pigs, enabled PRRSV prevalence in the pig population in Lithuania in which as much as 4.29 per cent of pigs under investigation had PRRSV-specific antibodies in 12 out of 30 Lithuanian regions, to be reduced tenfold.
2. By means of serological studies it was proved for the first time that PRRSV infection among wild boars in Lithuania was more prevalent than that on the pig-breeding farms.
3. It was for the first time that AT-nPGR with ORF5 and ORF7 oligonucleotide primers has shown that wild boars are active carriers of PRRSV of European genotype in Lithuania.
4. It was for the first time in the world that nucleotide sequences of ORF5 PCR product of wild boar PRRSV strains have been sequenced and publicised in GenBank database (No. KC714037-KC714042).

5. In the course of the investigations PRRSV strains of European genotype of subtypes 3 and 4 prevalent in wild boars, which have not been found in the pig population in Lithuania, were determined for the first time.

6. The investigations carried out provide the first evidence that wild boars can be a natural source of PRRSV in Eastern Europe.

**Practical significance of the study**

1. The latest research results of PRRSV infection prove that new pig-breeding technologies and conditions can reduce the prevalence of PRRSV in pig herds substantially.

2. By means of investigations it has been established that different diagnostic kits of the enzyme-linked immunosorbent assay INGEZIM PRRS EUROPA or IDEXX PRRS Herd Chek, which are used in routine diagnostics in many Lithuanian laboratories, are precise and reliable, and result differences do not exceed the allowable standards.

3. During the investigations it was tested and proved by the results that the commercial kits of the enzyme-linked immunosorbent assay could be used to determine PRRSV-specific antibodies in wild boar blood serum samples.

4. To determine wild boar PRRSV by means of AT-nPGR method ORF5 and ORF7 nucleotide primers were successfully tested and employed, which earlier were used to diagnose PRRSV strains circulating in the pig population.

5. Having proved for the first time that wild boars are a natural source of PRRSV infection in Lithuania, important data of the investigations, which allow exhaustive and long-term PRRSV prevalence and control studies to be included into the monitoring programme of such infectious diseases of wild boars as classical swine fever and Aujeszky’s disease, were submitted to the State Food and Veterinary Service.

6. The results of this work will help veterinary surgeons and specialists on pig-breeding farms to understand the PRRSV situation in the pig and wild boar populations in Lithuania better, to implement the preventative measures against PRRSV more successfully and receive better industrial results.

**MATERIALS AND METHODS**

**The subject of investigation**

The scientific research work done at the Laboratory of Immunology of the Lithuanian University of Health Sciences (LUHS), the Veterinary Academy, and the Serology Unit of the National Food and Veterinary Risk Assessment Institute between 2008 and 2012 was carried out following the approved requirements for work with animal samples: wild boar and pig blood serum and pathological material samples were collected from different localities in Lithuania, the quality of taking, storing, transporting the samples and data about collecting the samples was ensured. Blood serum samples, which were collected from 30 Lithuanian regions on 55 different pig-breeding farms, were used for studying PRRSV-specific antibodies: blood serum samples of sows and young pigs (n=895), boars (n=1682), sucking-pigs under three months of age (n=1385), and fattening pigs (n=4742). The following blood serum samples (n=1022) were collected for PRRSV infection studies from the boars accidentally shot during the autumn-winter hunting season between 2008 and 2011: blood serum samples of the young boars under 12 months of age (n=341), of the boars under 12 – 24 months of age (n=266), of 24-month old and older adult boars (n=379). Boar samples for studies were collected in all ten Lithuanian districts and in all 50 regions of Lithuania, in more than 300 different hunting sites.

To ensure reliability of the results of the comparative studies, blood serum samples (n=161) were used, which were collected from 12 regions of Lithuania, from 15 different pig-breeding farms, pig-breeding farms of other countries (n=110), where by means of the enzyme-linked assay PRRSV-specific antibodies and 18 control reference sera obtained from PRRSV reference R&D Laboratory, Deventer, Holland in 2011, while participating in international ring experiments, were detected.

**Detection of PRRSV-specific antibodies in serum samples**

To determine PRRSV-specific antibodies, the indirect method of enzyme-linked immunosorbent assay (ELISA) was used and the investigations were carried out by means of three commercial kits following the methodological instructions of the manufacturers: IDEXX PRRS HerdChek (IDEXX Laboratories, Maine, United States), IDEXX PRRS X3 (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland), INGEZIM PRRS Europe (INGENASA, Madrid, Spain).

The commercial kits used in the investigations were assessed taking into consideration the procedures for checking the ELISA method described by
Jacobson (1998). The control, reference or standard serum samples with PRRSV-specific antibodies and without them were selected for validation of the kits, the performance procedures were optimised using sera of different dilution or antibody level, and also, sensitivity and specificity were assessed, standard deviations and reproducibility of the research results were established.

All the schemes of the research methods of PRRSV-specific antibodies employed in the investigations were either accredited or verified according to the requirements of LST EN ISO/IEC 17025:2005 standard.

The research results were read with the help of a spectrophotometer (Tecan Sunrise) using a filter specified in methodology. The control and information reduction software “Magellan Standard” was used to measure the final points of all research results.

**Nucleic acid extraction and RT-PCR**

PRRSV RNA was detected in serum samples or lung tissue samples using Total RNA Prep Plus kit (A&A Biotechnology, Gdynie, Poland) or Trisol Reagent (Invitrogen Life Science) used according to the manufacturer’s instructions. For the reverse transcriptase polymerase chain reaction (RT-PCR), 5 µl of total RNA were used as a template. The reaction was performed using commercial RT-PCR reagents (Thermo Scientific) according to the manufacturer’s instruction and the previously described primers (Stadejek et al., 2002). A modified single-tube nested RT-PCR was performed according to Arūnas Stankevičius et al. (2005). ORF5 amplification was obtained with the second pair of primers, 606 bp long.

RT-nested PCR on ORF7 was performed using external (Oleksiewicz et al., 1998) and internal (Drew et al., 1997) EU-genotype primers. ORF5 NA-genotype corresponding primers described by A. Umthun et al. (1999b) and (Meulenberg et al., 1993) were used to amplify ORF5. RT-PCR products were purified using Nucleospin Extract II (Macherey-Nagel, Germany) commercial kit.

**Sequencing and phylogenetic analysis**

Purified, non-cloned ORF5 and ORF7 PCR amplicons were sequenced at the Sequencing Centre of the Institute of Biotechnology in Vilnius, Lithuania. Individual sequences of both strands of the ORF5 or ORF7 PCR products were determined with the same pair of primers used for nested PCR. Amplifying individual sequences of both strands of ORF5 and ORF7 PCR products were determined with fluorescent terminator BigDye Terminator v3.1 Cycle Sequencing Kit and further analysing products of purified samples with 3130xl Genetic Analyzer (Applied Biosystems). At least two different PCR products were sequenced to verify that no errors had occurred during DNA amplification and that the obtained sequences were correct. The sequences were assembled by using SeqMan program (Lasergene, program package, DNASTAR, Inc., Madison, USA).

The obtained sequences were compared with the reference set of the selected sequences from GenBank representing a full range of genetic diversity and geographic locations of EU-genotype PRRSV. All sequences were analysed in pair-wise comparisons. The referent Lelystad strain of the EU-genotype PRRSV was included into the comparisons. The sequences were aligned using the Clustal W software from MegAlign Lasergene software package. The same software was used for the alignment of the deduced ORF5 and ORF7 amino acids sequences and for construction of phylogenetic trees.

The phylogenetic tree was constructed by means of neighbour-joining methods using VR-2332, the prototype of the American genotype, as the outgroup strain. In order to assure statistical reliability of the trees, bootstrap values were calculated using CLC Gene Free Workbench software, with bootstrap values based on 100 analyses replicates (v4.0.01, CLC bio A/S, Aarhus, Denmark).

**PRRSV etalon strains and their amplification in MARC:145 cell culture. Immunoperoxidase monolayer assay (IPMA)**

Strains of the European genotype Lelystad and the United States genotype VR-2332 were used in AT-PGR, IPMA reactions. The highest dilution of PRRSV strains, which caused destruction of MARCKp145 cell layer or 50 per cent of the cells became specifically stained in the IPMA reaction, was defined by the PRRSV titre. The final PRRSV concentration for VR-2332 strain was $10^4$ TICD$_{50}$, and that for Lelystad PRRSV was $10^2$ TICD$_{50}$.

**Statistical analysis**

The data were processed by means of the method of descriptive statistics. The research results were evaluated using the statistics software package GraphPrism 3.0™. The 95 per cent confidence interval (CI) was calculated. The student’s confidence level and the results obtained were regarded to be statistically significant when $p<0.05$.

The CLUSTAL X program (Higgins et al., 1994) was used to carry out and compare the statistical analysis of nucleotide and predicted amino acid sequences. To construct a phylogenetic tree and to establish the bootstrap value NJ algorithm from the CLC Gene Free Workbench software (v4.0.1, CLC bio A/S, Aarhus, Denmark) was employed.
RESULTS AND DISCUSSION

Research results of PRRSV-specific antibodies in Lithuanian pig population in 2008–2011

The investigations into the prevalence of PRRSV carried out on the Lithuanian pig-breeding farms between 2008 and 2011 showed that 4.29% of the animals under investigation had antibodies specific to this virus. Attention should be drawn to the fact that by means of ELISA methods the unvaccinated piglets and pigs, which had no any clinical symptoms characteristic of PRRSV infection were investigated, and blood serum samples were accidentally collected for control investigations of other important infectious diseases. During the period under investigation PRRSV-specific antibodies were established in 2.76–8.15% of the blood serum samples of the pigs under investigation (Table 1), however, no statistically significant changes were established during that period.

Table 1. Research results of PRRSV-specific antibodies in pig blood serum samples collected in 2008–2011

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of pigs studied</th>
<th>Found positive</th>
<th>%</th>
<th>95 CI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>1548</td>
<td>73</td>
<td>4.72</td>
<td>3.28–6.44</td>
</tr>
<tr>
<td>2009</td>
<td>1210</td>
<td>71</td>
<td>5.87</td>
<td>4.41–7.36</td>
</tr>
<tr>
<td>2010</td>
<td>4743</td>
<td>131</td>
<td>2.76</td>
<td>1.92–3.6</td>
</tr>
<tr>
<td>2011</td>
<td>1203</td>
<td>98</td>
<td>8.15</td>
<td>6.99–9.31</td>
</tr>
<tr>
<td>Total:</td>
<td>8704</td>
<td>373</td>
<td>4.29</td>
<td>3.48–5.1</td>
</tr>
</tbody>
</table>

Having compared the obtained results with the results of the investigations into PRRSV infection carried out on stock-breeding farms in 2005–2007 (Stankevičienė et al., 2008), in which PRRSV antibodies were detected in up to 13.7% of blood serum samples, a significant decrease in the number of pigs infected with PRRSV was observed. Serological investigations carried out between 1998 and 2001, during which 29.2–40.7% of the PRRSV-positive samples were determined, confirm this tendency (Stankevičienė et al., 2002). Such a significant decrease in the number PRRSV-positive pigs can be accounted for by the fact that during the recent years the new owners have depopulated several large pig-breeding farms and at the present time the PRRSV-negative pig herds are raised there. Modern pig-breeding technologies have been commenced to be applied on the pig-breeding farms, paying great attention to the biosafety requirements, to putting pigs in quarantine, to grouping of pig and laboratory investigations. Live attenuated vaccines that have been used recently also prevent the outbreaks of PRRSV infections, significantly reduce the virus isolation into the environment and increase immunological resistance of the herd to PRRSV infection.

When assessing the PRRSV epidemiological situation in the Lithuanian pig population in the positive, the data of other countries should be taken into consideration too. They show that, for example, PRRSV-specific antibodies in Holland were determined on from 18 to 82% of pig-breeding farms (Duinhof et al., 2011), and in England, 39.8% of PRRSV-serologically positive samples of blood serum of the studied unvaccinated pigs of different age was established (Evans et al., 2008).

Our investigations into the prevalence of PRRSV carried out in different groups of pigs showed that PRRSV circulated more actively in the group of sows and young pigs (Table 2). During the investigations 16.98% of all the sows and young pigs under investigation (n=895) had PRRSV-specific antibodies, whereas the number of serologically positive samples in the group of boars and fattening pigs was considerably smaller (0.48–1.79%).

Table 2. Research results of PRRSV-specific antibodies in different groups of pigs

<table>
<thead>
<tr>
<th>Group of pigs</th>
<th>Number of studied pigs</th>
<th>Found positive</th>
<th>%</th>
<th>95 CI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sows and young pigs</td>
<td>895</td>
<td>152</td>
<td>16.98</td>
<td>15.98–17.98</td>
</tr>
<tr>
<td>Boars (of different age)</td>
<td>1682</td>
<td>8</td>
<td>0.48</td>
<td>0.03–0.93</td>
</tr>
<tr>
<td>Piglets under 3 months</td>
<td>1385</td>
<td>128</td>
<td>9.24</td>
<td>8.14–10.34</td>
</tr>
<tr>
<td>Fattening pigs</td>
<td>4742</td>
<td>85</td>
<td>1.79</td>
<td>0.95–2.74</td>
</tr>
<tr>
<td>Total:</td>
<td>8704</td>
<td>373</td>
<td>4.29</td>
<td>3.48–5.1</td>
</tr>
</tbody>
</table>

Having analysed the data of serological investigations of different years it became clear that the incidence of PRRSV infection in boars is very low not only when generally assessing the entire period of investigations but also the data of individual years show that only a small number (0.3–0.4%) of pigs belonging to that group had specific antibodies. This leads us to the conclusion that PRRSV infection practically does not spread through semen or through direct contact during mating on Lithuanian pig-breeding farms.

The results of our investigations also showed that the number of PRRSV-positive blood serum samples on small pig-breeding farms (up to 500 pigs) was reliably smaller than that on large or medium-sized farms. This leads us to the conclusion that concentration of pigs on large pig-breeding complexes create favourable conditions for PRRSV infection to
spread. The results of our investigation also clearly showed that up to 71.13 % of the unvaccinated PRRSV-positive pigs could be found in the complexes raising 15 000 – 30 000 pigs.

Investigations into PRRSV-specific antibodies on 55 pig-breeding farms in 30 regions of Lithuania showed that serologically positive samples were found on the pig-breeding farms in 12 regions of the country. In our opinion, these data also confirm a relatively favourable situation with respect to PRRSV infection in different regions of Lithuania because the greatest majority (60 %) of the pig-breeding farms in Lithuanian regions had no PRRSV-specific antibodies, which means, that PRRSV was not prevalent on those farms.

Research results of PRRSV-specific antibodies in Lithuanian wild boar population in 2008–2011

The data about the prevalence of PRRSV in the wild boar (Sus scrofa) populations are not abundant and those who are available are rather contradictory. It was only in 0.3 – 3.6 % of the cases in the United States, France and Germany, respectively, that PRRSV antibodies were detected in the population of wild boars (Saliki et al., 1998; Albina et al., 2000; Oslage et al., 1994). In the United States of America, no positive samples were determined in all the samples collected in 1976 and 1993, and only two positive animals were found in 1994 by Lutz and Wurm (1996). No PRRSV-serologically positive cases in 768 boar samples collected in 1992-1993 and 1995-1996, which were selected for the investigations, were determined in Germany either. In the neighbouring Poland, as well as in the Russian Federation, no PRRSV-specific antibodies were detected in the wild boar population (Fabisiak et al., 2013; Kukushkin et al., 2008). Samples of blood serum of wild boars were negative in Croatia and Slovenia (Vicente et al., 2002; D. Ruiz-Fons et al., 2006; Zupančič et al., 2002; Vengust et al., 2006). PRRSV was discovered in the wild boar lung tissue samples by means of polymerase chain reaction (PCR) in Italy and Germany (Bonilauri et al., 2006; Reiner et al., 2007). Investigations into the German wild boar population by molecular methods carried out by Reiner and others (2007) proved for the first time that PRRSV strains of the European (genotype 1) and the United States genotype could be directly determined by the reverse transcription polymerase chain reaction (RT-PCR) in 15.9 % of the lung tissue samples (Reiner at al., 2009). Also, recently one publication has been issued about the prevalence of PRRSV of genotype 2 in the population of wild hybrid pigs in China (Wu et al., 2011). The data about the prevalence of PRRSV infection in the wild boar populations is scarce in literature, whereas the results of our investigation provide new valuable epidemiological information about PRRSV infection in the wild boar population, which has not been extensively investigated thus far.

The results of our investigations carried out between 2008 and 2011 showed a comparatively large number of wild boars (6.36 %) that had PRRSV-specific antibodies (Table 3), and this number was even larger than the number of positive pigs serologically determined in the pig population (4.29 %). Moreover, this number of PRRSV-positive wild boar samples was larger than the number of PRRSV-positive wild boar samples obtained during the recent serological investigations carried out in Spain (2.0 – 3.0 %; Bodella et al., 2011; Closa-Sebastia et al., 2011), Germany (3.82 %; Kaden et al., 2009).

Table 3. Research results of PRRSV-specific antibodies in wild boar blood serum samples collected during the hunting season in 2008–2011

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of wild boars studied</th>
<th>Found positive</th>
<th>% 95 CI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>286</td>
<td>26</td>
<td>9.1</td>
</tr>
<tr>
<td>2009</td>
<td>274</td>
<td>15</td>
<td>5.5</td>
</tr>
<tr>
<td>2010</td>
<td>99</td>
<td>2</td>
<td>2.02</td>
</tr>
<tr>
<td>2011</td>
<td>363</td>
<td>22</td>
<td>6.06</td>
</tr>
<tr>
<td>Total:</td>
<td>1022</td>
<td>65</td>
<td>6.36</td>
</tr>
</tbody>
</table>

This conditionally large number of PRRSV-serologically positive animals can be accounted for by the favourable conditions, which have formed in the wild boar population in Lithuania. Density of wild boars increased considerably in Lithuanian forests during the period between 2008 and 2011 – from 1,84 to 2,66 wild boars per km². The prevalence of PRRSV in the wild boar population could have been determined by intensive migration and additional feeding during winter months. This determined an increase in the concentration of wild boars in certain places in the forest, which was conducive to the spread of PRRSV from one wild boar to another. The results of our investigations show that the spread of PRRSV among wild boars can be much more significant than it has been thought thus far, however, to prove that more thorough investigations into the wild boar populations in neighbouring Latvia, Belarus, and Kaliningrad Region are necessary. PRRSV serological investigations of wild boars in Lithuania are fruitful and they are the first investigations of this kind in Eastern Europe.

The research results of wild boars belonging to different age groups
carried out between 2008 and 2011 showed that PRRSV-specific antibodies in the samples of blood serum of adult (24 month-old) and older wild boars were determined more often than those in the group of the young or 12 – 24 months old animals (Table 4). In our opinion, this result shows that two-year-old and older wild boars can be the main source of PRRSV infection in Lithuania, however, this can conclusively be proved only having studied the clinical samples of wild boars by means of RT-PCR, which would enable cases of infection of this virus to be established directly. As far as we know, the only investigations in Europe and the world, which have directly proved the prevalence of PRRSV in the wild boar populations, were carried out in Germany (Reiner et al., 2007).

Table 4. Research results of PRRSV-specific antibodies in different age groups of wild boars

<table>
<thead>
<tr>
<th>Age group of wild boars</th>
<th>Number of wild boars studied</th>
<th>Found positive</th>
<th>%</th>
<th>95 CI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>The young under 12 months of age</td>
<td>341</td>
<td>9</td>
<td>2.6</td>
<td>2.6–7.2</td>
</tr>
<tr>
<td>Young 12-24 month old wild boars</td>
<td>266</td>
<td>8</td>
<td>3.0</td>
<td>3.2–6.9</td>
</tr>
<tr>
<td>24-month-old and older wild boars</td>
<td>379</td>
<td>48</td>
<td>12.7</td>
<td>10.4–14.9</td>
</tr>
<tr>
<td>Total:</td>
<td>1022</td>
<td>65</td>
<td>6.36</td>
<td>4.52–8.2</td>
</tr>
</tbody>
</table>

Wild boars that have PRRSV are spread over a large territory in Lithuania. This fact was confirmed by specific antibodies that were detected in the samples from 23 regions, which were attributed to 10 districts of Lithuania. This allows us to make the supposition that PRRSV did not get into the population of wild boars through accidental contact in some Lithuanian region or district. A wide distribution of serologically PRRSV-positive boar samples shows a regular circulation of this virus in the whole population of wild boars in Lithuania. The data of the investigations carried out by other authors show that a relatively small number of wild boars, which were in contact with PRRSV or detecting serologically altogether negative animals allows us to suppose that PRRSV infection practically either does not manifest itself in the population of wild boars or it manifests itself sporadically (Ruiz-Fons et al., 2007).

A large number of PRRSV-positive wild boars in Lithuania that we have established, which was even larger than that established in domesticated pigs, enables us to suppose that boars can be a natural source of PRRSV in Lithuania. This changes the prevailing opinion that there is no sufficient evidence about the PRRSV endemic source in the wild boar population (Meng et al., 2009). It should also be mentioned that wild boars are universally treated as an important source of infection for a classical swine fever virus or the Aujeszky’s disease virus, which can infect pigs very easily through direct contacts (Albina et al., 2000).

Results of the assessment (validation) of commercial ELISA kits in determining PRRSV-specific antibodies in blood sera of pigs and wild boars

Seeking to elucidate which most popular commercial kits intended for serological diagnostics by means of ELISA currently on the market can be suitable to determine specific antibodies of PRRSV strains of different genotypes, as well as seeking to evaluate sensitivity, precision and specificity of the kits when investigating samples of blood serum of not only pigs but also that of wild boars, a standard assessment (validation) of the methods for determining PRRSV-specific antibodies was made.

Table 5. Results of assessing homogeneity and stability of PRRSV-positive samples with the help of IDEXX HERDCHEK PRRS X3 kit

<table>
<thead>
<tr>
<th>Date of st.</th>
<th>01 17</th>
<th>01 17</th>
<th>01 17</th>
<th>01 18</th>
<th>01 18</th>
<th>01 19</th>
<th>01 19</th>
<th>01 19</th>
<th>01 19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stor. tem.</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Samples</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
<td>M/TK</td>
</tr>
<tr>
<td>KRa-23</td>
<td>3.270</td>
<td>3.623</td>
<td>2.901</td>
<td>3.401</td>
<td>2.969</td>
<td>3.420</td>
<td>2.800</td>
<td>2.822</td>
<td>2.980</td>
</tr>
<tr>
<td>KRa-26</td>
<td>2.271</td>
<td>2.621</td>
<td>2.126</td>
<td>2.541</td>
<td>2.169</td>
<td>2.621</td>
<td>2.226</td>
<td>2.130</td>
<td>2.216</td>
</tr>
<tr>
<td>KRa-28</td>
<td>2.437</td>
<td>2.444</td>
<td>2.444</td>
<td>2.589</td>
<td>2.353</td>
<td>2.444</td>
<td>2.248</td>
<td>2.174</td>
<td>2.053</td>
</tr>
<tr>
<td>KRa-29</td>
<td>1.775</td>
<td>2.052</td>
<td>1.836</td>
<td>2.164</td>
<td>1.754</td>
<td>2.052</td>
<td>1.812</td>
<td>1.809</td>
<td>1.854</td>
</tr>
<tr>
<td>KRa-30</td>
<td>2.631</td>
<td>2.554</td>
<td>2.481</td>
<td>3.165</td>
<td>2.571</td>
<td>2.484</td>
<td>2.481</td>
<td>2.437</td>
<td>2.517</td>
</tr>
<tr>
<td>KRa-31</td>
<td>2.205</td>
<td>2.299</td>
<td>1.999</td>
<td>2.417</td>
<td>1.843</td>
<td>2.109</td>
<td>1.985</td>
<td>1.816</td>
<td>1.942</td>
</tr>
</tbody>
</table>

The assessment of the homogeneity and stability of blood serum samples used for PRRSV serological investigations by means of ELISA showed that...
storing the samples at different temperatures prior to the tests did not influence the determination of PRRSV-specific antibodies to such an extent that they could change the research results significantly. Very similar results of the study of the samples were obtained because the confidence value (CV) of the standard deviation of the results of PRRSV-positive and PRRSV-negative serum samples did not exceed 5-10 % (Table 5). Also, the investigations showed that the samples remained highly stable when stored at different temperatures.

When carrying out serological PRRSV investigations by means of the enzyme-linked immunosorbent assay, using different groups of sera according to the established level of PRRSV antibodies, as well as working with both INGEZIM PRRS EU and IDEXX PRRS Herd Chek diagnostic kits, values of the results obtained by us corresponded with the original values.

The reproducibility of the results of the study of the samples made while working with three different diagnostic kits INGEZIM PRRS EU, IDEXX PRRS Herd Chek and IDEXX PRRS X3, using the selected PRRSV-positive samples of pigs and wild boars of the European genotype, as well as PRRSV-positive samples of the EU/US genotypes showed that the research result of both wild boars and pigs repeated themselves in carrying out all three different investigations and their results coincided. On the other hand, while working with INGEZIM PRRS EU and IDEXX PRRS X3 we managed to identify PRRSV-positive and PRRSV-negative serum samples received for comparative studies (CS) from the Reference R&D Laboratory (Deventer, Holland). When working with IDEXX PRRS X3, the conformity of our results with the results obtained in 48 laboratories in other countries accounted for 98.9 per cent, whereas the results obtained when working with INGEZIM PRRS EU diagnostic kit were identical to the results obtained in the Reference Deventer Laboratory. This allows us to draw the conclusion that all three commercial ELISA kits used in Lithuania are sensitive, specific and can be employed for serological PRRSV investigations not only into the pig but also into wild boar populations.

In summing up the results of the selected and reference serum studies it can be stated that commercial INGEZIM PRRS EU and IDEXX PRRS X3, IDEXX PRRS Herd Chek IFA kits evaluated by us are suitable for serological diagnostics in Lithuania when investigating samples of blood serum of both pigs and wild boars.

**Molecular research results of PRRSV strains of wild boars**

Scientific publications present very little information about direct PRRSV investigations into wild boars carried out by means of RT-PCR or other molecular methods. One case is described in Italy when PRRSV strains of genotype 1 were accidentally determined by means of PCR method in the lung tissue sample of a wild boar killed in a car accident (Bonlauri et al., 2006). PRRSV infection in the wild boar population was determined directly in Germany too, where strains of genotypes 1 and 2 were identified with the help of PCR (Reiner et al., 2009). No more cases of direct detection of PRRSV by both classical virusologic or immunologic methods were publicised, and this allowed us to make the supposition at the beginning of these investigations that PRRSV was not wide spread in the wild bore population and cannot be an important natural source of this infection for pigs, which is very characteristic of the case of classical swine fever or Aujeszky’s disease.

Scientific publications provide very limited information about a direct determination of PRRSV in the wild boar population by means of PCR method or any other molecular investigations, which clearly proves that this theme practically has hardly been investigated. The only publication in which the results of the investigations into the German wild boars is presented (Reiner et al., 2009) states that 6.2 % of the samples studied had the European genotype and 14.2 % of the samples had PRRSV strains of the United States genotype. Similarly as in Lithuania, in Germany, PRRSV RNA was much more often isolated from the lung tissue of wild boars than from the tonsils or blood serum. Contrary to our results, Kukushkin and others (2008) failed to detect PRRSV in the samples of the internal organs of the Russian wild boars by means of RT-PCR, and in Poland, molecular, as well as serological, investigations carried out showed that this virus was not prevalent in the populations of wild boars (Fabisiak et al., 2013).

The most significant and most valuable result of PRRSV molecular investigations into wild boars carried out by us from the scientific point of view was a successful amplification of the regions of ORF5 and ORF7 nucleotide sequences by means of RT-nPCR for which oligonucleotide primers that were used for molecular characterisation of PRRSV of pigs were employed and tested. Having for the first time employed specific oligonucleotide primers adapted to investigate pigs, we managed to obtain fragments of ORF5 and ORF7 nucleotide sequences of PRRSV strains of wild boars, which were successfully sequenced. It has been established that ORF5 encodes amino acid sequences of GP5 protein on the structure of which the response of the immune system of pigs to viral infection depends to a great extent. Contrary to ORF5 region, ORF7 region is very conservative and encodes nucleocapsid protein N, which is of vital importance to the virus. Nucleotide sequences that encode this protein are extremely stable and change very little but for each existing PRRSV subtype...
have a strongly expressed polymorphism of their size. Thus, products of ORF5 and ORF7 of RT-nPCR are of great importance to a molecular characterisation of PRRSV strains. In his only publication about PRRSV of wild boars Reiner et al. (2009) presented data from the conservative ORF1 region, which was not too suitable for a molecular characterisation of PRRSV strains because ORF1 nucleotide sequences were identical to many PRRSV strains of the same genotype and did not allow the strains to be attributed to some of the subtypes whose main nucleotide characteristics were described by Stadejek at al. (2006; 2008; 2013). Furthermore, he failed to obtain RT-nPCR products with oligonucleotide primers from ORF5 and ORF7 regions for the amplification of which other primers were used. Our investigations revealed clearly opposite results from those which have been known thus far, which means that classical oligonucleotide primers from ORF5 and ORF7 regions intended for pigs could be used for a direct determination of PRRSV strains of wild boars or a molecular characterisation of RT-nPCR or RT-PCR. Besides, it should be underlined that no data were publicised in scientific publications in the world prior to these investigations. Negative results of nested PCR with the primers of ORF7 region can be explained by PRRSV point mutation in some place of the primer hybridisation due to which the primer cannot connect and initiate synthesis of DNA fragment (Table 6).

Table 6. Results of investigations of wild bore lung tissue and blood serum samples carried out by means of AT-nPGR with ORF5 and ORF7 primers

<table>
<thead>
<tr>
<th>Wild boar sample</th>
<th>AT-nPGR results with ORF5 primers</th>
<th>AT-nPGR results with ORF7 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Num. studied</td>
<td>Num. positive</td>
</tr>
<tr>
<td>Blood serum</td>
<td>90</td>
<td>6</td>
</tr>
<tr>
<td>Lung fragment</td>
<td>105</td>
<td>13</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td><strong>195</strong></td>
<td><strong>19</strong></td>
</tr>
</tbody>
</table>

The phylogenetic analysis of ORF5 sequences of PRRSV strains showed that strains of two different subtypes were prevalent in the wild boar population in Lithuania (Fig. 1).

Fig 1. Distribution of wild boar PRRSV strains of subtypes 3 and 4 in Lithuanian districts

In the phylogenetic tree wild boar PRRSV strains grouped together with the reference PRRSV strains of subtypes 3 and 4 (Fig.4), which were determined in the pig populations in Belarus and Poland only. Wild boar PRRSV of subtype 3 prevalent in the Lithuanian wild boar populations were determined prior to the present investigations in Belarus only, and strains of subtype 4 were established in Latvia and Belarus (Fig. 2).

Wild boar PRRSV strains, which were identified in the course of the present investigation, are marked by arrows and shaded in the phylogenetic tree. The PRRSV strains, which belong to all four PRRSV subtypes of the European genotype, were used for the comparison of nucleotide sequences and construction of the tree. The letters LT stand for Lithuanian PRSSV strains, the letters LV indicate Latvian strains, Belarusian strains are marked with the letters BY and Polish strains are represented by the letters PL in the phylogenetic tree. The scale at the bottom of the phylogenetic tree indicates differences in ORF5 nucleotide sequences in terms of per cent.
the European PRRSV genotype (genotype 1) were prevalent in the pig population. In the ORF5 phylogenetic tree, pig PRRS viruses formed two separate branches of the phylogenetic tree the development scope of which was statistically significant (larger than 80). These research results coincided with the earlier data (Stankevičius et al., 2008; Čepulis et al., 2009) and therefore it can be supposed that PRRSV infection and the composition of the strains did not change and remained stable in the pig population.

ORF5 nucleotide sequences from six Lithuanian farms were similar to one another and formed a separate group of subtype 2 in the phylogenetic tree, which statistically significantly differed from the PRRSV strains of Western Europe. Taking into consideration the recently proposed classification (Stadejek et al., 2008), these strains can be attributed to PRRSV of subtype 2. The strains that belong to it clearly grouped with Belarusian and Russian PRRSV in the ORF5 phylogenetic tree.

It is known that a certain amino acid sequence expressing N-glycosylation sites of GP5 protein encoded by ORF5 is the main mechanism enabling viruses to avoid the impact of the immune system and persist in the organism for a long time blocking, reducing or completely preventing specific neutralising antibodies from affecting viruses (Ansari et al., 2006). Neutralising PRRSV antibodies ineffectively combine with glycosylated PRRSV GP5 protein (Ansari et al., 2006; Faaberg et al., 2006). Taking into consideration the fact that GP5 is a very important protein for immunogenicity, during the investigations N-Glycosylation sites, which localised at 37, 46 and 53 amino acid positions for Lithuanian pig and wild boar strains were determined.

These data were in line with the data of the investigation into the strains prevalent in Belarus that was carried out by T. Stadejek and others (2006), however, none of ORF5 wild boar or pig nucleotide sequences had lost N-glycosylation site at amino acid position 46. Contrary to these results, PRRSV strains, which had lost N-glycosylation site at this position, were possible to determine on two farms in Belarus.

Summing up the research results it can be stated that those PrRSV strains of subtypes 3 and 4, which are not found on pig-breeding farms, are prevalent in the Lithuanian wild boar population. This can cause a serious danger to those farms because the strains of subtype 3 are noted for high pathogenicity (Karnichiuk et al., 2010). There are no data about pathogenicity of the strains of subtype 4 in scientific publications because this PRRSV was detected on one farm of Belarus only (Stadejek et al., 2006) and in several pig-breeding complexes in Latvia (Čepulis et al., 2009).

**Fig. 2. Phylogenetic analysis of PRRSV strains prevalent in pig and wild boar populations in ORF5 region.**

**Molecular research results of PRRSV strains in the pig population**

Our investigations also enabled the PRRSV strains, which currently circulate in the Lithuanian pig population, to be established. In the course of the five recent years pig breeding technologies, pig breeds, the PRRSV epidemiological situation have changed in essence therefore these investigations allowed us to evaluate certain peculiarities of PRRSV infection and changes in the strains anew. Our research results obtained showed that earlier identified strains of subtypes 2 and 1, which belonged to
Different PRRSV subtypes in Lithuanian wild boar and pig populations allow us to suppose that PRRSV is an endogenous infection of wild boars and can be a natural source of PRRSV infection for domesticated pigs. This supposition based on the investigations carried out is supported by the fact that wild boars can be carriers of other dangerous viruses, such as viruses of swine fever, Aujeszky’s disease, which have been recognised to be an important sources of infection for domesticated pigs for a long time.

Our results substantiate the opinion that wild boars are a natural source of PRRSV infection in Eastern Europe.

**CONCLUSIONS**

1. By means of PRRSV serological investigations carried out on the Lithuanian pig-breeding farms between 2008 and 2011, it was established that 4.29 per cent (95% CI 3.48–5.1) of the pigs under investigation in 12 of 30 Lithuanian regions had antibodies specific to this virus.

2. PRRSV-specific antibodies were determined in 16.98 per cent (95% CI 15.98–17.98) of blood serum samples of sows and young pigs and these results statistically significantly exceeded the data of serological investigations into boars (0.48 per cent; 95% CI 0.03–0.93) and fattening pigs (1.79 per cent; 95% CI 0.95–2.74).

3. The established statistically significant difference between the number of PRRSV-positive samples on large pig-breeding farms raising 15 000–30000 pigs and small (up to 500 pigs) farms proves that concentration of pigs in modern Lithuanian pig-breeding complexes create favourable conditions for PRRSV infection to spread.

4. Investigations into PRRSV infection carried out between 2008 and 2011 showed, for the first time in Eastern Europe, a relatively large number, 6.36 per cent (95% CI 4.52–8.2), of wild boars that had antibodies specific to this virus, which was even larger than the number of serologically positive pigs determined in the pig population on the Lithuanian pig-breeding farms.

5. The largest number of PRRSV-specific antibodies (12.7 per cent; 95% CI 10.4–14.9) was determined in the group of 24-month-old and older adult wild boars, which was significantly larger than the number of positive samples detected in the group of the young (2.6 per cent; 95% CI 2.6–7.2) or 12–24-month old animals (3.0 per cent; 95% CI 3.2–6.9).

6. Determining PRRSV-specific antibodies in wild boar samples collected in 23 Lithuanian regions belonging to 10 districts presents the first preliminary proof that wild boars can be a natural source of PRRSV infection in Eastern Europe.

7. The evaluation of different commercial ELISA kits when investigating reference and clinical blood serum samples of pigs and wild boars shows that they are precise, sensitive and reliable in determining PRRSV-specific antibodies not only in pig but also in wild boar samples, and the established differences of the results are statistically insignificant.

8. In Lithuania, 8.2 per cent of wild boars are active carriers of PRRSV of the European genotype and their cDNA can be identified by means of RT-nPCR with both ORF5 and ORF7 oligonucleotide primers, which are used in routine diagnostics of PRRSV in many laboratories of the European countries.

9. ORF5 nucleotide sequences obtained from Lithuanian wild boars are arranged in the phylogenetic tree on the branches of two clearly expressed phylogenetic groups (the third and the fourth subtypes), which had a significant bootstrap value and differed from the PRRSV strains prevalent in the pig population in Lithuania where PRRSV strains of subtypes 2 and subtypes 1 were established.

**LIST OF PUBLICATIONS**


CONFERENCES


buvo nustatytas 2008 metais ir siekė 9,1 proc. (95 proc. CI 7,3–10,8), o mažiausias 2,02 proc. (95 proc. CI 0,5–3,5) buvo nustatytas 2010 metais. Panašios studijos JAV, Prancūzijoje ir atitinkamai Vokietijoje (Salici ir kt., 1998; Albina ir kt., 2000; Oslage ir kt., 1994) parodė, kad lauksių šernų populiacijoje KRKSV antikūnų buvo rasta tik 0,3–3,6 proc. atvejų. Kaimyninėje Lenkijoje, o taip pat Rusijos Federacijoje specifinių antikūnų KRKSV šeruose nebuvo nustatyta (Fabisiak ir kt., 2013; Kukushkin ir kt., 2008).

Tyrimų rezultatai skirtingoše šernų amžiaus grupėse 2008–2011 metais parodė, kad suaugusių 24 mèn. ir vyresnių šernų kraujo serumuose buvo nustatyti dažniausiai teigiami antikūnai, nes jie buvo gauti tik augintinių sëktu sezono auxiliary ir augintinių šernųviešose paauglies sëktuose. Taip pat buvo atrinkti kontroliniai, referentiniai ar standartiniai šernų kraujo serumes, atliekant standartinës populiacijos nustatymo metodą (standardaus serumes nustatymo metodą). Dabar dauguma publikacijose išbandyti kiaulių KRKSV padermių molekulinės analizės metodus, pavyzdžiui, ORF4 antikûnių nustatymo metodą (validacija). Daugelio ankstesnių imunofermentinës analizës metodų tyrimai parodė, kad šie metodai gali bûti pakankami jautrus ir specifinës. Taip pat svarbu buvo išsiaiškinti, kurie šiuo metu rinkoje esantys populiauriais serologinei diagnostikai imunofermentinës analizës (IFA) metodo skirti komerciniai rinkiniai gali bûti tinkami nustatant skirtinës genotipës KRKSV padermių specifinës antikûnus, o taip pat, norint įvertinti kiaulių kraujo serumu, tiksliai atsikartotų metu kiaulių, bet ir šernų kraujo serumo mëginiai, buvo atlikti standartinës serumes nustatymo metodų įvertinimas (validacija). Daugelio ankstesnių imunofermentinës analizës metodo tyrimų rezultatai (Cho ir kt., 1996; Denac ir kt., 1997; Seuberlich ir kt., 2002; Takikawa ir kt., 1996; Witte ir kt., 2000) jëro, kad šis metodas gali bûti pakankami jautrus ir specifikas KRKSV padermių tyrimams, tačiau dauguma publikacijose minëtų metodų nebuvo visapusiškai įvertinti ir palyginti tarpusavyje. Taip pat svarbu buvo išsiaiškinti, kurie tyrimų rezultatus gali ëkoti mëginës homogeniškumo bei stabilumo parame, ir ar pakankamai tikslus kiaulëms skirti IFA rinkiniai, nustatant KRKSV specifinës antikûnës dësnius kraujo serumas.

Tyrimuose buvo panaudoti šiuo metu Lietuvoje KRKSV antikûnëms tirti skirti komerciniai rinkiniai IDEXX PRRS herdChek, IDEXX PRRS X3, INGEZIM PRRS Europa, kurie buvo ëvertinti atsižvelgiant į Jacobson (1998) aprašytas IFA metodo patikrinimo procedûras. Rinkinių validacija buvo atrinkti kontroliniai, referentiniai ar standartiniai serumų mëginiai su KRKSV specifinës antikûnës ir be jës, buvo optimizuotos atlikimo procedûros, naudojant įvairių skiedių ar antikûnų lygmens serumas, taip pat, ëvertintas jautrumas bei specifika, nustatytos standartinës populiacijos kintamumas ir tyrimo rezultatų atkartojami.

KRKSV serologijos tyrimams IFA naudojamos kraujo serumo mëginiai skirti komerciniai rinkiniai IDEXX PRRS herdChek, IDEXX PRRS X3, INGEZIM PRRS Europa, kurie buvo ëvertinti atsižvelgiant į Jacobson (1998) aprašytas IFA metodo patikrinimo procedûras. Rinkinių validacija buvo atrinkti kontroliniai, referentiniai ar standartiniai serumų mëginiai su KRKSV specifinës antikûnës ir be jës, buvo optimizuotos atlikimo procedûros, naudojant įvairių skiedių ar antikûnų lygmens serumas, taip pat, ëvertintas jautrumas bei specifika, nustatytos standartinës populiacijos atkartojami.
regiono, kuris nėra labai tinkamas molekuliniam KRKSV padėmių
charakterizavimui, nes daugeliu to pačio genotipo KRKSV padėmės
ORF1 nukleotidų sekos yra identiškos ir neleidžia padarmo priskirti kuriam
nors subtipui, kurių pagrindines molekulinės charakteristikas aprašė
Stadejek ir kt. (2006; 2008; 2013). Be to, jam nepavyko gauti AT-nPGR
produkų su oligonukleotidiniais pradmenimis iš ORF5 ir ORF7 regionų, kurių
pagauninimui buvo naudojami kitos padarmos. Mūsų atlikti tyrimai
atskleidė aiškiai priešingus rezultatus nei iki tol buvo žinomu, kurie reiškia,
kad šermų KRKSV padėmių tiesioginiam nustatymui ar molekuliniams
charakterizavimui AT-nPGR arba AT-PGR1 gali būti naudojami klasikiniai,
kai jie skirti oligonukleotidiniais pradmenimais iš ORF5 ir ORF7 regionų. 
Pasaulioje tėvų šimtų, kurių atliko mokslinės publikacijos duomenų nebuvo
paskelbta. Līdzinės PGR neigiamus rezultatus su ORF7 srities padarmo
galima paaiškinti atsiradusiu KRKSV taškine mutacija kuroje nors padarmo
hibridinimo vietoje, dėl ko padarmo negali prisijungti ir įnicijoti DNR
fragmento sintezą.

KRKSV padėmių ORF5 seko filogenetinė analizė parodė, kad Lietuvoje
šermų populiacijoje yra paplitusios dvių skirtų subtipų padarmos. Šermų
KRKSV padėmės filogenetinėje medžje grupavosi kartu su referentinėmis 3-ojo ir 4-ojo subtipo KRKSV padėmėmis, kurios buvo
nustatytos tik Baltarusijos ir Latvijos kiaulių populiacijose. Lietuvos šermų
tarpe paplitusios KRKSV 3-ojo subtipo padarmo iki šių tyrimų nustatytos
tik Baltarusijoje, o kiaulės subtipo padarmo Latvijoje ir Baltarusijoje.

Mūsų atlikti tyrimai taip pat leido nustatyti ir KRKSV padarmu, kurios
pastaruoju metu mėgina Lietuvos kiaulių populiacijos. Rezultatai
parodė, kad kiaulių tarpe padarmo anksčiai identifikuotos 2-ojo ir 1-ojo
subtipų padarmos, kurios priklauso europietiškajam KRKSV genotipui. 
ORF5 filogenetiniame medžje kiaulių KRKSV virusai suformavę dvi
atskirą filogenetinio medžio šakas, kurių pločio dydis buvo statistiškai
reikšmingas (didesnis už 0.05). Šie tyrimai atlikęs padaugėjo su ankstesniais
duomenimis (Stankevičius ir kt., 2008; Čepulis ir kt., 2009) ir todėl leidžia
manoti, kad KRKSV infekcija ir padarmų sudėtį kiaulių tarpe nepasikeité
eišliko stabili.

Atsižvelgiant į tai, kad ORF5 koduojamas GP5 yra labai svarbus
baltymas imunosystemy, tyrimų metu buvo nustatyta N-glikozilacinio
vietos, kurios Lietuvos šermų ir kiaulių padarmos laikotarpiu buvo pavandenytos
ties 37, 46 ir 53 aminorūgščių padarmo. Čiems šiemis atsiradęs, kurių pastaruoju metu
neįprastai sudaryti kiaulės padarmo, o dėl to padarmas labai išplėtė
ir išliko stabili.
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