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**Improvement of the diagnostics of PRRS,  
assessment of the prevalence, and genetic characterisation  
of the Hungarian strains**

**Brief version of the PhD thesis**

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## Introduction

Porcine respiratory and reproductive syndrome (PRRS) is a widespread disease of swine characterised by reproductive disorders in gilts and sows and by respiratory signs, leading to death mostly in neonatal, suckling and weaned piglets. The disease was first recognized and described in the late '80s in the United States. In Europe the first outbreak was reported in North Western Germany in 1990 and the disease spread throughout Europe soon after. The first Hungarian seropositive herds were found in 1996, whereas the first isolation of the virus was performed in 1999.

The porcine respiratory and reproductive syndrome virus (PRRSV) is an enveloped, single stranded RNA virus of the *Arteriviridae* family, member the order *Nidovirales*. The genome of PRRSV is approximately 15 kilobases in length, and comprises nine open reading frames (ORFs).

Various sequence analyses have proven that there are marked genetic differences between the two major genotypes, the European and North American strains identical only in 60% at nucleotide level. In the past, European isolates were considered to be less variable than the American strains, however, recently significant differences were found among the European type strains leading to the definition of four subtypes.

In the diagnostics several direct and indirect methods are used. Direct methods detect the virus itself, its genetic material, or the viral proteins, whereas indirect techniques measure antibodies produced by host immune system in response of the infection. Commercially available indirect ELISA assays are suitable for the assesment of the immunoprofile of a herd, however they do not indicate the actual level of protection, can show up as negative in persistently infected pigs, and due to the relative high rate (~1%) of fals positive reactions the results are reliable only in case of extensive positivity. Because PRRSV is one of the most genetically variable viruses and the molecular diagnosis of the disease constitutes a great challenge, it is crucial to develop fast, specific, sensitive methods that are prone to detect strains belonging to both genotypes, and all European subtypes.

As the first step of the research our goal was to detect the viruses circulating in different pig herds. For this purpose we aimed to develop an RT-PCR assay that is capable of detecting every possible PRRSV strain by the amplification of the conserved part of its genome (ORF7). After that samples we wanted to sequence the variable part of the genome (ORF5) on the positive with a second round of RT-PCR by the use of other set of primers. We aimed then to determine the phylogenetic relations of our PRRSV strains by the comparison

of these sequence data to each other and to sequences downloaded from the GenBank (Bethesda, USA, [www.ncbi.nih.gov](http://www.ncbi.nih.gov)). We wanted to find out if Type-2 strains are also present in Hungary as it was observed in some neighbouring countries (Slovakia, Austria), and if so they are wild type, or vaccine-like strains. In addition we aimed to collect strains from neighbouring countries that do not have published PRRSV sequences in the GenBank to compare them to Hungarian strains, and to describe their phylogenetic relations to each other and to sequences downloaded from the GenBank.

In the case of viruses changing so rapidly, like PRRSV the continuous improvement of the diagnostic methods is crucial, concerning especially the molecular diagnostic methods in order to make them capable of detecting strains of the given period, moreover new variants that are constantly arising due to the continuous genetic drift. Nevertheless those PCR techniques are coming more and more to the front that, in addition to yes/no results provided by the conventional gel-based assays are able to quantify the nucleic acid copy contents of the analysed samples. Thus, the aim of the third step of our research was to develop a new, robust, fast, reliable, and specific real time molecular amplification method, that is able to detect and quantify not only every present PRRSV strain but even those that are going to arise in the future.

## Materials and methods

In the first phase of our research we collected samples from large scale Hungarian pig herds. Those herds were targeted that had been proven to be PRRS positive by serological analysis in other institutes, or showed symptoms resembling to PRRS such as reproductive disorders, high mortality rates among the suckling piglets, or respiratory disorders affecting young animals. Sera samples were collected from sows and piglets showing symptoms of PRRS, and lung, peribronchial lymphnode and tonsil samples were collected from deceased animals and aborted foetuses. Sera samples were collected and analysed from herds in Serbia as well, with histories of increased respiratory symptoms.

Based on sequence data downloaded from the GenBank different primer sets were created for diagnostic and phylogenetic purposes. For the diagnostic PCR reactions primers were designed to anneal to the most conserved parts of the genome (ORF7 and 3'NCR) and amplify a 393nt long fragment in case of the European, and a 428nt long fragment in case of the American type strains. For the phylogenetic purposes 2-2 genotype specific primer pair were designed to amplify a variable part of the genom, the ORF5. The amplicons obtained by the phylogenetic reactions were subjected to direct nucleotide sequencing from both directions. The sequence data were then aligned to each other and to the most important sequences available in the GenBank, phylogenetic calculations were performed, putative amino acid sequences and potential N-linked glycosylation sites were determined.

In the next phase of our work we developed a novel real time quantitative RT-PCR method. For the design of the primers and the probe 235 PRRSV sequences were downloaded from the Genbank. The position of the forward primer was determined on the 3' end of ORF6, whereas the probe and the primer were designed to bind next to each other on the 5' end of ORF7. The sequence of the probe was identical to the corresponding region of the Lelystad virus' sequence, whereas multiple nucleotide degenerations were incorporated into the primers according to the alignments.

To assess the sensitivity and the efficacy of the assay 10 fold dilutions were prepared from known amounts of target RNA obtained from the Lelystad virus, the Belarus Soz-8 strain (member of the European subtype 3), and the American type attenuated vaccine strain (Ingelvac® MLV) by the use of MEGAscript® T7 Kit. For the preparation of the RNA samples conventional, gel based RT-PCR reactions were performed with the forward and the unlabeled form of the reverse primer. The forward primer was modified by a specific T7

promoter sequence that was added to its 5' end. The gel-purified amplicons were then transcribed to RNA by the use of the Kit according to the manufacturer's recommendations. The RNA concentrations of the undiluted samples were determined by a Nanodrop ND 1000 instrument, and ten-fold dilutions were prepared from each sample from a  $10^{11}$  to  $10^0$  RNA copy number. The results of the test of the ten-fold RNA dilutions were used to calculate the slope of the standard curves.

The sensitivity was also evaluated using serial dilutions of the Lelystad virus and the P129 isolate propagated on PAM and MA-104 cell cultures, respectively. Ten-fold dilutions were prepared in PBS (phosphate buffer saline) beginning from cell culture supernatants containing  $10^5$ TCID<sub>50</sub>/ml virus. Melting point analysis of the amplicons was performed in the end of each run to confirm the specific amplification of the examined virus' nucleic acid.

Specificity was tested using our PRRSV field strain collection obtained from field cases. Selected swine pathogens were also tested with the system to exclude the cross reactivity of the PRRSV PriProET. Included in these were porcine circovirus type 2, swine influenza virus (H3N2, and H1N1), classical swine fever virus, porcine respiratory corona virus, Aujeszky's disease virus, porcine parvovirus, porcine cytomegalovirus.

Five PRRSV negative, conventional 4 weeks old piglets were infected intranasally with 2-2ml supernatant, containing  $10^4$ TCID<sub>50</sub>/ml of a Hungarian wild type virus. Blood was collected on day 1, 2, 3, 4, 5, 6, 7 PI. On the eighth day the pigs were euthanized and tonsil and lung samples were obtained, and together with the sera samples they were subjected to real-time PCR. Three animals were inoculated with virus free supernatant, and involved in the study as negative controls.

## Results

Altogether we performed the ORF5 sequence analysis of 45 Hungarian (obtained from 25 herds), and 8 Serbian PRRSV strains. By comparing these sequences to each other and the most important sequences downloaded from the GenBank it can be concluded, that the majority of the Hungarian sequences (42) similarly to the Western European sequences belong to the European genotype, subtype 1. Within the subtype the Hungarian strains can be clustered 4 monophyletic subgroups: two of them is formed by vaccine-like, and the other two is formed by wild-type strains. The biggest subgroup contains half of the European type Hungarian sequences (21). Within this subgroup the separation of the strains was in correlation with the distance of the geographical location of the herds where these samples were collected. Three sequences were clustered in the American genotype. Two of them were originated from the same herd, and shared 90-91% nucleotide identity with the Canadian “Quebec” reference strain, whereas the other one was related to an attenuated live vaccine virus strain that is widely used in Europe, but not authorised in Hungary.

We identified nine vaccine like strains, that were obtained from vaccinated herds, but from non-vaccinated animals, and showed high nucleotide identity to the sequence of the vaccine used in the herd. Analysing the nucleic acid and the deduced amino acid identity values of the vaccine related strains compared to live vaccine virus strains it is remarkable that in case of the derivatives of one of the commercially available vaccine, all amino acid changes were found in the putative ectodomain, consistently at the same amino acid positions. Analysing the putative N-linked glycosylation sites of the first ectodomain of the live vaccine virus strains and their derivatives, it was found that almost all the vaccine-like variants lost the N-46 glycosylation site (compared to the vaccine strain). All these sequences were recovered from aborted fetuses, or carcasses having severe respiratory problems prior to death. In an other herd where the other vaccine was used the AA changes of the vaccine like strains were found in random distribution, all these like sequences were obtained from clinically healthy animals, and such losses were not observed within the herd.

The ORF5 analysis of the 8 Serbian PRRSV sequences revealed, that they belong to the European genotype, subtype 1, and show high similarity to each other and to sequences found in Denmark.

During the development of the PriProET-based real-time quantitative assay, the sensitivity of the system regarding the number of nucleotide copies was evaluated by using

known amounts of recombinant viral RNA obtained from the Lelystad, the Soz-8, and the Ingelvac MLV® strain. The detection limit was ten copies of viral RNA in the reaction mixture and the assay allowed linear detection in the range of  $10^0$  to  $10^{11}$  RNA copies/reaction. The standard curves were obtained with the Lelystad and the Ingelvac MLV® strain. All reactions were performed in triplicate, and the mean data of cycle threshold was used to design the standard curves. The PCR efficiency (E) was 0.99 in case of the Lelystad virus, and 1.00 in case of the the Ingelvac MLV® strain, whereas the correlation efficient ( $R^2$ ) was 0.99 in both cases.

In case of the diluted Lelystad and P129 cell culture supernatant the detection limit was one TCID<sub>50</sub>/ml virus which, according to the appropriate standard curves, was equal to 61 and 42 copies of viral RNA, respectively. The assay could detect each strain that was proven to be positive by gel-based PCR. Reactions performed with other (non-PRRSV) viruses gave negative results.

PRRSV RNA appeared in the sera of the inoculated animals on day 2 post infection (PI), and after a slight, non-significant decrease the maximum quantities were observed on days 6 and 7 PI when  $8-9 \times 10^5$  RNA copy/ml was present in the samples. The tonsil samples contained  $1-2 \times 10^5$  RNA copy/tissue g, whereas the lung samples contained  $6-9 \times 10^8$  RNA copy/tissue g. Melting point analysis performed after each PCR confirmed that the amplicons obtained from the experimental samples had the same, specific  $T_m$  (76.0°C) as the challenge virus, confirming the successful inoculation of the animals. The negative control animals had no viral RNA in their blood or organ samples.



## Discussion

The aim of the first step of our research was to collect samples from Hungarian PRRS infected farms, and to try to assess the prevalence of PRRS infection. By combining the data of three diagnostic institutes it can be concluded that even though the prevalence of the disease in our days (around 10%) is higher than before, the situation in Hungary is still better as it can be observed in some Western European (Denmark, The Netherlands), and even in neighbouring countries (Austria), where the prevalence can be more than 50%.

Detailed examination of the samples collected from pig farms in the last five years revealed that the large majority of the Hungarian samples belong to the European subtype 1. Monophyletic subgroups are also formed by these sequences, one of which comprises half of the strains. Within this large subgroup the different branches are formed by sequences originating from the same geographic area. Because of the fact that these herds are not in close proximity to each other, the airborne aerosol transmitted infection can be excluded. The source of infection among these herds might have been the introduction of infected animals from the other herds, the transport of infected pigs on roads close to the animals, improper disinfection of vehicles (food transporting trucks) that enter to the farms, or even workers wearing contaminated clothes.

The alignment of the putative amino acid sequences, and the analysis of the N-linked glycolisation revealed, that almost every Hungarian PRRSV strain gained a new glycolisation site compared to the Lelystad virus, due to a Asp→Asn change, thus the large majority of our strains had 3 potential glycolysation sites. The same change was observed in case of other European strains as well.

Unique clusters were formed by vaccine-like sequences. We found vaccine-like sequences in non vaccinated animals living in vaccinated herds in the case of both commercially available attenuated vaccines. In one case after application of a wrong vaccination protocol (two times switching from one vaccine to the other) one of the vaccines (vaccine B) could be demonstrated from young animals even 3-4 months after re-introduction of the other vaccine (vaccine A). By the examination of these sequences and comparing them to the corresponding region of the original vaccine we observed consistent AA changes at the 37., 46., 59., and the 60. nucleotide positions, and all the vaccine-like variants lost the N-46 glycosylation site, by a consistent Asn→Lys AA change. Comparing the nucleotide and the amino acid sequence alignments it is also remarkable that the incidence of the synonymous

mutations in case of the vaccine-like strains is quite low. One might speculate that the explanation of this phenomenon could be the selective pressure of the immune system directed against the GP5 ectodomain, and indicates putative positive and negative selection sites on this part of the genome as observed previously by other investigators. To avoid this selective pressure, those variants that have developed greater phenotypic differences (AA changes) compared to the original strain, had better chance to spread and persist within the herd while silent mutations were not rewarded by higher chances to multiply and spread.

We found three strains that were clustered among the American strains. Two of them showed 90% nucleotide identity with the Canadian reference strain. The origin of these strains remains unclear, since no direct, or indirect contact was reported with Canadian pigs. Vaccine like American strain was also found in Hungary, but even though we do not have information about the history of the herd, it can be postulated that the virus was originated from a Western European country, where these strains are endemic in the pig herds.

The aim of the second step of our research was to develop a real-time reverse transcriptase polymerase chain reaction method that is fast, sensitive, and – unlike other methods using hydrolysing (TaqMan) probes – is able to tolerate mismatches in the probe region. The latter is the keypoint of this assay, since PRRSV is one of the most variable and rapidly evolving RNA viruses.

The analysis of the serial dilutions of known amounts of target RNA revealed that our PriProET assay is able to detect low amounts of template nucleic acid. The PriProET method is proven to tolerate at least five mismatches in certain regions of the probe binding site (Ingelvac MLV®) without significant loss of sensitivity, and PCR efficiency compared to the perfect matching Lelystad strain. The melting point of the amplicon with five mismatches is high (66.8°C), referring to a relative strong connection between the probe and the target genome. This might indicate that this PriProET method has the potential of tolerating even more mismatches in the probe binding site.

Melting point analysis performed directly after the PCR could confirm the specific hybridisation of the different PRRSV strains. By comparing the melting point of the amplicons to the perfectly matching Lelystad strain, mutations can be predicted before sequencing, however mutations at the 5' end of the target region will have less influence on the melting point. Although the exact number of mismatches can not be stated by the degree of the temperature decrease, according to the sequences deposited in the GenBank Type 1 and Type 2 strains can be differentiated prior to sequencing the amplicons. However the probe binding site is relatively conserved, and – especially it's 5' end – is characteristic of the

PRRSV stains within a genotype (Type 2 strains have at least 4 mismatches due to a deletion and 2 conserved mutations), in the future it can not be excluded that Type 1 strains with 4, 5 or even more mismatches may arise showing same melting points as Type 2 strains.

The analysis of samples obtained from experimental infections revealed that in an acute PRRSV infection the primary replication occurs in the lungs, containing 1000 times more PRRSV RNA than the tonsil or the sera samples. PriProET can be used for following up the dynamics of the viral RNA quantities in experimental animals, and melting point analysis can confirm the specificity of the reaction by comparing the  $T_m$  of the amplicons to that of the challenge virus.

Summarising the results obtained with phylogenetically distinct PRRSV strains, and experimental samples, it can be concluded that the PriProET is a robust and sensitive method that is proven to tolerate numerous target-probe mismatches making it a reliable tool to detect and quantify PRRSV.

## New scientific results

- 1) To detect PRRSV infection a novel gel based RT-PCR method has been developed to amplify the conserved part of the genome (ORF7, 3' NCR), that is proved to be able to detect strains of both genotypes, and due to the different size of amplicons it can be used to the preliminary differentiation between the genotypes.
- 2) For sequence determination and phylogenetic analyses gel based RT-PCR has been developed to amplify the variable part of the PRRSV genome the ORF5.
- 3) Numerous Hungarian PRRSV ORF5 sequences were determined and phylogenetic analyses were performed for the first time.
  - a) It was found that most of the PRRSV sequences we analysed (93,33%) belonged to the European genotype, subtype 1.
  - b) We reported the presence of American type strains in Hungary for the first time, We found both wild type and vaccine like strains as well.
  - c) The ORF5 sequence of a wild type american PRRSV strain was first determined in Europe.
  - d) The alignment of the deduces amino acid sequences revealed, that large majority of the Hungarian sequences gained a new N-linked glycolisation site compared to the Lelystad virus on the 37. amino acid of the GP5.
  - e) In case of one of the commercially available attenuated vaccine virus consistent changes were observed suggesting strong selective pressure on the first ectodomain of the GP5. These vaccine-like variants lost a glycolisation site at the 46. amino acid. (The same was not observed in case of the other attenuated vaccine available in Hungary.)
- 4) The ORF5 sequence of Serbian PRRSV strains were determined for the first time, and it was found that they belonged to the European subtype 1, and show high similarity to each other and to strains isolated in Denmark.
- 5) A novel primer-probe energy transfer based real-time quantitative RT-PCR method has been developed for the detection of PRRSV.
  - a) The assay was proved to be able to detect, and quantify phylogenetically distinct PRRSV strains.
  - b) Melting point analysis of the amplicons was found to be suitable for the detection of mismatches on the probe binding site.

- c) Sequence analyses, and the examination of serial dilutions prepared from known amounts of recombinant RNA copies of different PRRSV strains revealed that the assay could tolerate at least five mismatches in certain regions of the probe binding site without significant loss of sensitivity, and PCR efficiency.

## **List of publication related to the thesis**

**Balka Gyula**, Rusvai Miklós, Kecskeméti Sándor, Kiss István: PRRS - újabb kihívás előtt a sertéságazat : 1. A vírus sajátosságai : Irodalmi összefoglaló és saját tapasztalatok. Magyar Állatorvosok Lapja, 2006. 128, 524-532.

**Balka Gyula**, Rusvai Miklós, Kecskeméti Sándor, Kiss István: PRRS - újabb kihívás előtt a sertéságazat. 2. A betegség járványtani, kórtani és immunológiai sajátosságai. Irodalmi összefoglaló. Magyar Állatorvosok Lapja, 2008. 130, 31-38.

**Gy. Balka**, Á. Hornyák, Á. Bálint, I. Kiss, S. Kecskeméti, T. Bakonyi, M. Rusvai: Genetic diversity of porcine reproductive and respiratory syndrome virus strains circulating in Hungarian swine herds. Veterinary Microbiology 2008. 127, 128-135.

**Gy. Balka**, Á. Hornyák, Á. Bálint, Zs. Benyeda, M. Rusvai: Development of a one-step real-time quantitative PCR assay based on primer-probe energy transfer for the detection of porcine reproductive and respiratory syndrome virus. Journal of Virological Methods, accepted for publication.

## **List of presentation and posters related to the thesis**

**Gy. Balka**, Á. Hornyák, Á. Bálint, I. Kiss, S. Kecskeméti, M. Rusvai (poszter): Genetic diversity of vaccine origin a wild type Porcine Reproductive and Respiratory virus strains circulating in Hungarian swine herds. 5<sup>th</sup> International Symposium on Emerging and Reemerging Pig Diseases, 24-27 June 2007, Krakow, Poland. In: Proceedings of the 5<sup>th</sup> International Symposium on Emerging and Reemerging Pig Diseases p.: 179.

**Gy. Balka**, Á. Hornyák, T. Barna, A. Farsang, G. Kulcsár, M. Rusvai (poszter): Transmission of a modified live virus vaccine from vaccinated to nonvaccinated pigs. Vaccine Congress, 9-11 December 2007, Amsterdam, The Netherlands In: Proceedings of Vaccine Congress p. 153.

**Gy. Balka, Á. Hornyák, Á. Bálint, M. Rusvai** (előadás): Development of a one-step real-time quantitative PCR assay based on primer-probe energy transfer for the detection of porcine reproductive and respiratory syndrome virus. EuroPRRSnet Workshop, 24-25 July 2008, Bruxelles, Belgium

**Gy. Balka, Á. Hornyák, Á. Bálint, M. Rusvai** (poszter): Development of a one-step real-time quantitative PCR assay based on primer-probe energy transfer for the detection of porcine reproductive and respiratory syndrome virus. 5<sup>th</sup> International PRRS Symposium, PRRSV Reverse Genetics: From the Laboratory to the Field, 2008. December 5-6, Chicago, USA In: Proceedings of the 5<sup>th</sup> International PRRS Symposium p. 50.

### **List of publications in referred papers**

T. Németh, N. Solymosi, **Gy. Balka**: Long term results of subtotal colectomy for acquired hypertrophic megacolon in eight dogs. Journal of Small Animal Practice 2008. 49, 618-624.

Jakab Csaba, Kovács Rita Beáta, Szabó Gyula, **Balka Gyula**: Negyedik agykamrai plexus chorioideus papilloma nyolcéves tibeti mastiffban: Klinikopatológiai esetismertetés. Magyar Állatorvosok Lapja, 2004. 126, 743-749.

Pétsch Márta, Jakab Csaba, **Balka Gyula**, Vörös Károly, Manczur Ferenc: Glomerulonephritis következtében kialakult pulmonalis thromboembolia. kutyában: Esetismertetés Magyar Állatorvosok Lapja, 2005. 127, 428-435.

Jakab Csaba, Bánky Áron, Kincses Krisztina, **Balka Gyula**, Demeter Zoltán: Kutyák bördaganatainak előfordulási gyakorisága és hisztopatológiája. Magyar Állatorvosok Lapja, 2006. 128, 140-149.

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