FP7 Collaborative Project n°245141:

“New tools and approaches to control Porcine Reproductive and Respiratory Syndrome in the EU and Asia”

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Final Report

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Executive Summary (1p. max.)
Since its discovery in the late 1980s, porcine reproductive and respiratory syndrome virus (PRRSV) has emerged as one of the most prevalent infectious diseases of pigs worldwide. It is a positive-sense, single-stranded RNA, enveloped virus classified within the genus Arterivirus. Two genotypes (1 and 2) are recognised that originally were described as European and American respectively because of the geographic origin of their prototypic strains. Genotype 1 is further separated into three subtypes and even a fourth one appears to be present (Stadejek et al., 2013, Vet Microbiol). Due to its extensive genetic variability, highly pathogenic PRRSV strains causing extensive pig mortality such as high fever disease in China, atypical PRRS in America and subtype 3 Eastern-European strains, have emerged. PoRRSCon is an initiative of 15 partners, originating from Europe and Asia, with strong expertise in virology and immunology. The aim of this project was to develop new tools and approaches to control PRRS. As a new tool to monitor and control emerging strains, our Polish partner (NVRI), an OIE reference laboratory for PRRS, created a public virus bank (http://www.porrsccon.ugent.be) that included European, Asian and American isolates encompassing both genotypes. Recent sequencing data generated by the consortium indicated that only genotype 1, subtype 1, isolates appeared to be present in Western and Central Europe. A comparison of commercial real-time RT-PCR kits now on the market suggests that if an introduction of highly pathogenic and highly diverse Eastern European subtypes 2-4 would occur in Western Europe, it could be undetected by routinely applied RT-PCR methods, mostly validated using subtype 1 strains. Therefore, the combination of several techniques (such as serological methods, shown to be satisfactory in proficiency tests, and RT-PCR based on multiple primers/probes) that should be constantly validated according to the currently circulating strains, is recommended to assure optimal diagnosis of PRRSV infections. A molecular epidemiology study of the isolates from within a country showed that in Europe, PRRSV (recently) crossed borders via trade of semen or live animals, which is important information for the development of national control measures. Furthermore, a limited degree of cross protection/cross reaction between subtype 1 and subtypes 2-4 isolates makes it necessary to test the cross antigenicity/neutralising capacity of any new vaccine candidates prior to marketing. The causes of the higher pathogenicity of some PRRSV isolates were investigated. Enhanced inflammatory immune response, higher induced apoptosis, downregulation of cell surface molecules, a stronger down-regulation of canonical pathways such as the interplay between the innate and adaptive immune responses, cell death and TLR3/TLR7 signalling, the use of (an) alternative entry receptor(s) contributing to wider tropism, as well as different immunobiological characteristics may cause the observed higher pathogenicity of subtype 3 strains compared to subtype 1 strains (Morgan et al., 2012, Vet Microbiol; Weesendorp et al., 2012, Vet Microbiol; Weesendorp et al., 2013, Vet Microbiol; Frydas et al. 2013, Vet Res; Badaoui et al., 2014, PLoS One), while Nsp9 and Nsp10 together contributed to the fatal virulence of HP-PRRSV in China (Li et al., 2014, PLOS Pathog). Concerning vaccine development, both B and T-cell epitopes were characterized (Vanhee et al., 2011, Vaccine; Mokhtar et al., 2014, Vaccine). The efficacy of innovative, autogenous inactivated PRRSV vaccines was assessed against homologous and heterologous challenges. Inactivated farm-specific PRRSV vaccines can be useful tools to boost PRRSV-specific humoral immunity in sows and reduce viremia in weaned piglets (Geldhof et al., 2012, BMC Vet Res en 2013, Vet Microbiol). Finally, significant progress was achieved in the development of attenuated marker HP-PRRSV vaccines that fully protect piglets from lethal challenge by highly pathogenic PRRSV and allow DIVA (Wang et al., 2013, Vet Res). Technical achievements of the project that will assist scientists worldwide, include: (1) the development of fast and robust methods for long range RT-PCR amplification (Kvisgaard et al. 2013, Virol. Methods), (2) a technique using next generation sequencing to sequence PRRSV directly from primary material/cell culture without any prior sequence knowledge (Lu et al. 2014, Virol J), (3) the knowledge that monocyte-derived macrophages can be used as a PRRSV infection model instead of alveolar macrophages, reducing the number of pigs required for the collection of cells (Garcia-Nicolas et al., 2014, Virus Res.), (4) an advanced protocol proposed to test the efficacy of PRRSV vaccines (Karniychuk et al., 2012, Therioigenology), (5) a published general review on virus inactivation (Delrue et al., 2012, Expert Rev Vaccines), and (6) released new PRRSV sequence data. Because PRRSV, endemic in Europe, has spread to many other countries, only an international control strategy may bring efficient solutions to the PRRS problem. With the support of the European Commission, the PoRRSCon consortium was initiated, consisting of European and Asian PRRS experts that are in contact with national and international authorities, farmer organisations, and PRRS experts in the USA. As attested by 75 peer-reviewed scientific publications and numerous dissemination activities worldwide, the knowledge generated has significantly contributed to the final aim of the project, the development of new tools and efficient approaches to control PRRS.
Summary description of the project context and the main objectives (4p. max.)

PRRSV is the major cause of reproductive and respiratory problems in pigs worldwide. It is endemic in Europe and other parts of the world. It was estimated in the United States that losses due to PRRSV cost the pork industry USD 664 million per year (Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers, MCHC, Holtkamp et al, 2013, Journal of Swine Health and Production). During an outbreak in The Netherlands, a mean loss of €126 per sow was observed (Nieuwenhuis et al., 2012, Vet Rec). High abortion rates, mortality of pre-weaned piglets and respiratory disease in fattening pigs are the main features of this syndrome. Controlling this disease is a main priority in pig producing countries. PRRSV is a positive-sense, single-stranded RNA, enveloped virus classified within the genus Arterivirus. Nowadays, two genotypes are recognised (type 1 and type 2) that originally were described as European and American because of the geographic origin of their prototypic strains. The European genotype is further separated in three recognized subtypes (1 - 3) and even a fourth subtype appears to be present (Stadejek et al., 2013, Vet Microbiol). It has been clearly shown that the best protection is induced when the vaccine virus belongs to the same genotype. Due to its high genetic variability, highly pathogenic strains of this RNA virus have emerged, causing up to 20 - 30 % mortality (such as high fever disease in China, atypical PRRS in America and disease caused by subtype 3 European strains). These new strains are difficult to control by presently available vaccines. PRRS is also undermining the pig's immunity, leading to extensive bacterial infections in PRRSV infected pigs. Antibiotics are consequently intensively used in order to control these bacterial infections, leading to antibiotic resistance. The only sustainable defence will be the development of adaptable, inactivated, vector or attenuated marker vaccines, which can safeguard the pig industry and animal welfare for the future.

PoRRSCon is an initiative of 15 partners from Europe and Asia with strong expertise in virology and immunology. The aim of this project was to develop new tools and approaches to control PRRS. To reach this final goal, the following specific objectives (SO) were set up for this project:

- SO1: Characterise genetically and antigenically current PRRSV isolates in Europe and Asia;
- SO2: Have a better understanding of the complex pathogenesis of PRRSV with different clinical outcome (tissue distribution, target cells, kinetics), immunological response and immune modulation,
- SO3: Find the genetic basis of pathogenicity/virulence of highly virulent PRRSV strains;
- SO4: Identify the PRRSV proteins and domains on these viral proteins that are involved in the induction of the immunity against PRRSV and in the immune modulation of PRRSV;
- SO5-7: Develop new generation, adaptable, efficacious and safe marker vaccines that can be adapted to new emerging PRRSV strains. Different approaches including inactivated vaccines (SO5), subunit marker vaccines (SO5), vectored marker vaccines (SO6) and attenuated marker vaccines (SO7) will be tested.
- SO8: Develop DIVA assays that allow to differentiate infected from vaccinated animals.

To achieve these main objectives, the PoRRSCon project was divided into 10 Work Packages (WPs), including a management WP, and each WP had its own objectives

• WP 1: Management
• WP 2: Collection bank and harmonisation: (1) creating a European and Asian central collection bank for virus isolates and other PRRSV-related biological material, (2) standardization and harmonization of protocols and (3) performing ring trials.
• WP 3: Genotyping and serotyping of PRRSV isolates/strains: (1) To evaluate the genetic diversity of archival and recent PRRSV isolates, (2) To investigate the level of cross-neutralization between selected PRRSV genotypes
• WP 4: Pathogenesis and virulence: (1) Comparative analysis of the pathogenesis of PRRSV strains in order to understand differences in pathogenicity/virulence, (2) Identification of the viral gene(s) involved in the virulence of highly pathogenic PRRSV strains causing porcine high fever disease (PHFD), (3) Identification of transcripts induced by highly virulent and low virulent PRRSV strains, (4) Identification
of viral ligand(s) interacting with sialoadhesin, viral counterpart(s) for CD163 and protease cleavage sites on viral proteins

- **WP 5: Immunobiology of PRRSV**: (1) Analyse the interaction of PRRSV with innate immune cells and the indirect effects on adaptive immune cells, (2) Analyse mechanisms of immune evasion initiated by PRRSV infection, (3) Identify viral targets for protective T cell responses

- **WP 6: Development of inactivated vaccines**: (1) Development and testing efficacy of inactivated vaccines against PRRSV strains that are isolated in vaccinated herds, (2) Development and testing efficacy of inactivated vaccines against highly virulent East-European and Asian PRRSV strains, (3) Development of subunit vaccines using the baculovirus system, (4) New strategies to target viral antigens to DC

- **WP 7: Development of recombinant marker vector vaccines**: (1) Development of vaccines based on rTGEV vectors, expressing GP5/M or GP2a/GP3/GP4, (2) Improvement of immune responses against PRRSV by engineering of the viral inserts and the co-administration of genetic adjuvants, (3) Clone PRRSV GP5 and M genes and modified forms thereof into replicon vectors, and packaging into viral replicon particles (VRP) for delivery to and expression of the encoded proteins in dendritic cells (4) Provide prototype vaccines for further in vitro screening and in vivo efficacy studies in pigs (WP9), (5) Determine the capacity of the replicon to support other PRRSV genes such as GP2, GP3 and GP4, as well as multiple gene sequences, for future applications, (6) In vitro screening, selection and in vivo testing of GP5/M or GP2a/GP3/GP4 constructs

- **WP 8: Development of attenuated marker vaccines**: (1) Attenuation of PRRSV by reverse genetics, (2) Introducing a marker in attenuated vaccines allowing differentiating infected and vaccinated animals (DIVA)

- **WP 9: In vitro and in vivo testing of preselected candidate vaccines**

- **WP10: DIVA serological assays**: (1) Production of recombinant PRRSV proteins and monoclonal antibodies to be used in DIVA serological assays and design of DIVA serological assays, (2) Test DIVA serological assays with sera from work packages 6, 7 and 8

In the next section, WP9 and WP10 will not be described separately, but included in WP6, WP7 and WP8.

**Description of the main S & T results/foregrounds (25p. max.)**

**WP2 Collection bank and harmonisation, Leader: Dr. Katarzyna Podgórska, NVRI (Poland)**

*Task 2.1 Central European and Asian collection bank.*

During the realization of the project, extensive sampling in East European countries as well as EU bordering regions was undertaken. Based on collected samples, a unique bank of PRRSV strains was established, including isolates and samples collected from pigs infected with strains classified in type 1 (subtypes 1, 2, 3 and tentative subtype 4) from Central and Eastern Europe as well as type 2 strains from USA, Europe and high pathogenic PRRSV strains from Vietnam. A central database of information on PRRSV strains and other related biological material available in the partners’ laboratories was created and updated. At the end of the project, the database included information on more than 170 PRRSV strains and samples from experimental infection studies, monoclonal antibodies, paraffin-embedded tissues, polyclonal sera, antigens, and plasmids carrying modified PRRSV genes. Most of the PRRSV strains were made available, on request, for institutions not participating in the project via the public section of the project website (http://www.porrscon.ugent.be). To facilitate information retrieval and exchange of biological materials between the project partners, the PRRSV information database was accessible via the wiki section (access restricted to the consortium) of the PoRRSCon website. Selected PRRSV positive samples (48 isolates, 90 tissue and 5 serum samples) from Polish, Belarusian, Lithuanian, Ukrainian and Latvian pig farms or wild boars were submitted for whole genome sequencing. The results of sequencing reported in other WPs provided valuable information about the genetic variation and evolution of PRRSV:
- No evidences for the presence of highly diverse PRRSV subtypes from East European countries in Europe were found.
- Two subtype 2 isolates from Belarus were used for the first time in inoculation study linked to the NADIR project (DTU-VET and NVRI, project n° FP7-228394, "Porcine reproductive and respiratory syndrome virus (PRRSV): Virulence of East-European atypical isolates") that provided unique tissue/monovalent sera collection. The aim of this study was to compare the virulence of subtype 2 strains with classical strain from subtype 1. Overall, obtained results indicated that the level of virulence of some strains belonging to subtype 2 may be higher compared to classical European strains and similar to previously reported for strains of subtype 3.
- A reference set of samples representing all known PRRSV subtypes was assembled and used for proficiency tests and evaluation of diagnostic methods (see Tasks 2.2 and 2.3).

**Task 2.2 Standardization and harmonization of protocols.**
Using the reference panel of samples created in Task 2.1, the evaluation of (1) serological and (2) RT-PCR diagnostic methods was performed:
(1) The sensitivity and specificity of ten different commercial and in house ELISA kits was evaluated. The comparison of the serological tests indicated that **most of the serological methods characterized PRRSV strains with satisfactory performance**. No significant decrease of sensitivity regarding highly diverse Eastern European PRRSV subtypes was observed. However, some problems with specificity of the kits concerning sera collected in specific herds were identified.
(2) Seven commercial real-time RT-PCR assays were compared. In this case, **significant differences regarding qualitative diagnostics as well as analytical sensitivity were observed between the real-time RT-PCR assays**, especially regarding diverse subtypes 2-4 from the East European countries and high pathogenic PRRSV strain from China. These findings emphasize that continuous monitoring of PRRSV genetic diversity, evaluation of diagnostic tests and updating primers and probes sequences are necessary to allow proper diagnostics. Close cooperation between diagnostic laboratories and the manufacturers of diagnostic PCR kits would be beneficial for both sides.

Based on the protocols submitted and validated by the PoRRSCon partners at different stages of the project, a Standard Operating Procedures (SOPs) book was prepared and distributed between the partners. Two major parts included 12 protocols aiming at virus isolation, detection and characterisation as well as detection of antibodies specific to PRRSV.

Detailed characterisation of PRRSV circulation in 22 Polish farrow-to-finish farms was undertaken to develop a consistent diagnostic strategy for PRRS. Herds differed regarding the quantity of sows (from 70 to 1400 heads), pig flow systems and applied PRRS control measures. In total, 3610 serum samples (70 to 574 samples per farm) were tested individually with **in house** indirect or differential ELISA (as in Stadejek et al. 2007). Pooled samples were examined with commercial real-time RT-PCR. Additionally, the performance of RT-PCR and **in house** ELISA adapted to oral fluid samples was analysed based on 20-30 samples of oral fluids collected in each farm. A highly variable picture was observed in analysed herds. Even within one farm and one age group, the virological and serological status of pigs between different pens was often different. Based on the obtained results, it can be concluded that a complete picture of PRRSV circulation in a herd can be obtained only by testing large number of animals by serological and PCR methods. However, examination of oral fluids indicated that **oral fluid testing by ELISA can be a cost effective alternative to serum analysis for a pen based diagnosis**. Comparison of RT-PCR results obtained in sera and oral fluids were non-consistent, what suggests that modifications of protocols and/or addition of sample stabilizers may be necessary.

**Task 2.3 Ring trials**
Two Proficiency Tests were organized to evaluate the ability of participating laboratories to detect PRRSV specific antibodies (Serological Proficiency Test) and viral RNA (RT-PCR Proficiency Test). The aim of Proficiency Tests was to identify problems in the areas of RT-PCR and serological diagnostic methods in the laboratories of the partners. A unique reference panel of samples developed in Task 2.1
including highly diverse East European strains of different subtypes (genotype 1) and genotype 2 strains (including a high pathogenic strain) was used. In the first Proficiency Test, 10 participants used 17 different RT-PCR methods and 7 participants submitted results of 8 ELISA assays and 2 immunoperoxidase monolayer assays (IPMA). In the second Proficiency Test, 8 laboratories tested samples with 11 diverse RT-PCR assays while 7 different ELISA kits and 1 IPMA protocol were applied by 7 participating laboratories. The results of the organized proficiency tests supported conclusions drawn based on comparison of commercial serological and RT-PCR assays. The analytical and diagnostic sensitivity of RT-PCR methods were highly variable, especially in case of East European strains of type 1 subtypes 2-4. Presently, there are no evidences of the presence of those subtypes in Central and Western Europe. However, the results of Proficiency Tests and comparison of commercial real-time RT-PCR kits suggest that if an introduction of East European subtypes would occur, it could be undetected by routinely applied RT-PCR methods that were mostly validated based on strains from subtype 1. Therefore, the combination of several methods is recommended to assure optimal diagnosis of PRRSV infections. Moreover, applied methods should be constantly validated according to the currently circulating strains. Considering the high genetic variability of PRRSV, the development and application of methods utilizing multiple primers/probes would be recommended. More consistent results were obtained in the serological Proficiency Tests. The results of the immunoperoxidase monolayer assays (IPMA) and ELISA tests proved that all applied protocols were able to detect specific antibodies in most of the samples, including antibodies directed to subtypes 2 and 3 of genotype 1.

WP3 Geno- and serotyping of PRRSV isolates/strains, Leader: Dr. Lars E Larsen, DTU VET (Denmark)

Task 3.1 Genetic diversity of archival and recent PRRSV isolates
Development of methods:
In the frame of this project, several new and innovative methods were developed for full-length sequencing of the PRRS virus genome for different applications.

- Fast and robust methods for long range RT-PCR amplification and subsequent next generation sequencing (NGS) were developed and validated on nine Type 1 and nine Type 2 PRRSV viruses (Kvisgaard et al. 2013, Virol. Methods). The methods generated robust and reliable sequences both on primary material (lungs, nasal swabs and serum) and cell culture (MDCK) adapted viruses and the protocols performed well on all three NGS platforms tested (Roche 454 FLX, Illumina HiSeq2000, and Ion Torrent PGMTM Sequencer). These methods will greatly facilitate the generation of more full genome PRRSV sequences globally. Up to now, these protocols have successfully being used to generate full length PCR products for more than 40 PRRSV isolates and 10 of these have been successfully completely sequenced using either the Illuminia or the FLX platform with a coverage of close to 100%.

- Another technical achievement was the development of a method using Next Generation Sequencing to sequence PRRSV directly from primary material/cell culture without any prior knowledge of the sequence. This method was used to sequence the Olot/91 strain grown on macrophage and its variants identified in cell culture (Lu et al. 2014, Virol J.). The advantages of this method are that no prior knowledge of the target sequences is required, it is possible to do multiplexing of strains and it allows identification of “natural” variants/quasispecies. The disadvantage is that high RNA quality is required and that high (85%) host RNA contamination makes it necessary to do in vitro amplification and sucrose cushion purification prior to the analysis. This technique should enable the detection of low-frequency nucleotide variants and hence provides a snapshot of the microevolution in the entire viral population. These protocols will assist scientist throughout Europe to make full length characterization of local PRRSV isolates which in turn will be to the benefit of PRRSV scientists, diagnosticians and decision makers in Europe also beyond the present project.

Sequences:
The focus of WP3 was to gain an extensive overview of the diversity of PRRSV in Europe and Asia. In the frame of the project, a total number of partial sequenced isolates added up to an impressive 958 ORF5 and 429 ORF7 sequences representing isolates collected in Poland, Vietnam, China and 7 West-
European countries. In addition, using innovative methods for full genome sequencing developed by the project partners, a total of 114 PRRSV isolates have been fully sequenced and much more are in the pipeline (Kvisgaard et al. 2013, J. Virol. Methods, Virus Res and Vet Microbiol; Lu et al. 2014, Virol J).

**Analysis of sequences:**
- The ORF 5 of the PRRSV viruses from a total of 101 PRRSV samples from eight European countries (Denmark, Belgium, UK, Italy, Spain, Poland, The Netherlands and France) were analysed phylogenetically. Overall, the identity between isolates was 80-100% at the genetic level. The national diversity varied among the countries, but this may merely reflect the differences in time and interval of collection. The phylogenetic analysis performed showed that all isolates belonged to genotype 1. In general, isolates from within a country clustered together in the phylogenetic tree, but several countries had isolates that clustered in distinct clusters. Interestingly, some clusters contained nearly identical viruses isolated in different countries. These findings indicate that PRRSV (recently) crossed borders via trade of semen or live animals. However, the high number of national clusters also indicates that, in general, the national PRRSV populations in the different European countries are separated. In four different countries, ORF5 sequences very closely related to the Lelystad vaccine virus were identified. This may indicate that the vaccine strain had spread from herd to herd, that animals in the herd were vaccinated at the time of sampling or that the Lelystad-like lineage of viruses has persisted in several countries.

- In addition to the diversity of Western European PRRSV isolates, a total of 39 Central East (CE) European strains from Poland, Hungary, Romania and Croatia were analysed. All of the CE isolates belonged to Type 1, subtype 1 confirming that the subtypes 2-4 have still only been found in countries east of Poland (Baltic Lands, Russia and Belarus) and have not (yet) been introduced into the rest of Europe.

- Also, 20 isolates collected from 7 provinces in Vietnam were sequenced (ORF5 and ORF7) and analysed phylogenetically. The analysis showed that all of the Vietnamese strains belonged to genotype 2 and there was a high level of similarity among the Vietnamese strains (ORF7: 98.9-100%) except for one strain (97.8 – 98.7%). This could be indicative of more than one introduction of PRRSV into Vietnam and ongoing sequence analysis of further genome regions is in progress to verify this. The full genome analysis of 10 Vietnamese strains confirmed the ORF5 results and also confirmed that all strains had the 30aa deletion in NSP2, the hallmark of the Chinese strains causing severe disease throughout Asia for the last years.

- The diversity of PRRSV in China was assessed based on NSP2, ORF5 and ORF3 sequencing. The 30aa deletion in NSP2 was present in a majority of Chinese HP-PRRSV but was not always present and novel deletion/insertion patterns have now been found in recent strains collected from 128 clinical samples originating from 17 regions of China between 2008 and 2012.

- Sequence information on the ORF5 and ORF7 are important for the prediction of the antigenic variation of circulating PRRSV isolates and, consequently, forms an important basis for the selection of vaccines strains. The variability of other parts of the genome may, however, also play an important role in the pathogenesis and epidemiology of PRRSV, but full-length sequence data were only available for a few European PRRSV strains at the beginning of the project. By using the method developed in the project, the genome of more than 100 European strains and 10 Vietnamese strains has now been fully characterized. Phylogenetic analysis of the full genome sequences generally confirmed the diversity established by analysis of ORF5, but other interesting features became evident when more or all PRRSV genes were included in the analysis. Recombination is currently recognized as a factor for the high genetic diversity of PRRSV but the frequency of such recombination events and the genome segments involved are not well known. We examined published ORF5 sequences and full-length genome sequences generated in Spain and Denmark within the scope of the project in order to determine potential recombination breakpoints along the viral genome (Martin-Valls et al. 2014, J Virol). The results indicated that most of the ORF5 datasets contained at least one recombinant sequence. **When complete genome sequences were examined, both genotype 1 and 2 sets presented breakpoints** (10 and 9, respectively), resulting in significantly different topologies before and after the breakpoints. **Mosaic**
genomes were detected in genotype 1 sequences. These results may have significant implications for the understanding of the molecular epidemiology of PRRSV.

Task 3.2. Cross-neutralisation between selected PRRSV isolates
Monospecific sera has been generated by experimental challenge against a range of different PRRSV strains representing all subtypes of genotype 1 PRRSV. A limited cross-neutralisation activity was found between sera raised against the subtype 3 strain Lena and the more common “Lelystad-like” subtype 1 virus (Karniyichuk et al. 2010, BMC Veterinary Research). Further investigations into the basis of these differences in reactivity between isolates were performed. Monoclonal antibodies (Mabs) were generated and characterised against synthetic overlapping linear dodecapeptides. These studies showed that 12 of the Mabs recognised linear peptides in GP3 and GP5 proteins whereas the remaining three Mabs recognised non-linear regions in the N protein. GP3-, GP5- and N-specific mAbs were further tested for their reactivity with antigenically different PRRSV isolates. The GP3-specific antibodies recognized all European isolates tested and one of the American isolates, although reactivity with the Belgian isolate 94V360 was weak. The GP5-specific antibodies only recognized PRRSV LV and showed no reactivity with any other PRRSV isolate tested. This was surprising, since two other European type PRRSV isolates included in the analysis, 94V360 and 07V063, showed the exact same amino acid sequence at the antigenic region recognized by the mAb (Van Breedam et al. 2011, Vet Immunol Immunopathol).

These results further confirm findings from the study of Martinez-Lobo and co-workers (2011) that showed no correlation between the sequences of predicted epitopes, glycosylation and the neutralizing phenotypes despite a clear clustering of the isolates into categories according to kappa values.

The results of the experimental studies performed within this project have shown that there is a limited degree of cross protection/cross reaction between Western European subtype 1 isolates and subtypes 2-4 isolates circulating in Eastern Europe. This has to be considered when the efficacy of new vaccines is assessed. The results of the neutralization studies confirm that it is not possible to predict the neutralising phenotype of a given PRRS virus or vaccine based on genetic data. Thus, it is necessary to test the cross antigenicity/neutralising capacity of any new vaccine entities prior to marketing.

WP4 Pathogenesis and virulence, Leader: Dr. Annemarie Rebel, DLO-CVI (The Netherlands)

Task 4.1. Differences in virus replication kinetics, target cells, cytokine profiles and immune response between PRRSV strains with different pathogenetic/virulence potential

We compared the immunological and pathological responses of pigs after infection with European PRRSV subtype 3 strains causing overt clinical signs (Lena, SU1-Bel; Karniyichuk et al., 2010, J Gen Virol) in comparison to European PRRSV subtype 1 strains (Lelystad-Ter Huurne (LV), Belgium A (strain 07V063), Britain (215-06)) causing subclinical infections. Two animal experiments were performed, one at AHVLA and one at CVI (described in Weesendorp et al., 2012. Vet Microbiol and Morgan et al., 2012 Vet Microbiol). One virus strain, the Lelystad-Ter Huurne (LV) strain was used in both studies at the two different locations to make results comparable.

At the CVI, the subtype 3 strain Lena was used, and compared with the subtype 1 strains LV and Belgium A. Sixteen pigs were inoculated per strain, and sixteen pigs with PBS. At days 7 and 21 post-inoculation (p.i.), four pigs per group were immunized with an Aujeszky disease vaccine (ADV) to study the immune competence after PRRSV infection. Infection with the Lena strain resulted in fever and clinical signs. This was not observed in the Belgium A or LV-infected pigs. Infection with the Lena strain resulted in high virus titers in serum, low numbers of IFN-γ secreting cells, a change in leukocyte populations and a delayed antibody response to immunization with ADV. Levels of IL-1beta, IFN-alpha, IL-10, IL-12, TNF-alpha and IFN-γ mRNA of the Lena-infected pigs were increased during the first week of infection. For pigs infected with the Belgium A or LV strain, the effects of infection on these parameters were less pronounced, although for the Belgium A-infected pigs, the level of the analysed cytokines, except for TNF-alpha, and leukocyte populations were comparable to the Lena-infected pigs.

At the VLA, piglets were infected with 3 strains of PRRSV-I: LV, 215-06 a British field strain and SU1-bel from Belarus. Post-mortem examinations were performed at 3 and 7 days post-infection (dpi), and
half of the remaining animals in each group were inoculated with an Aujeszky’s disease (ADV) vaccine to investigate possible immune suppression resulting from PRRSV infection. The subtype 3 SU1-bel strain displayed greater clinical signs and lung gross pathology scores compared with the subtype 1 strains. This difference did not appear to be caused by higher virus replication, as viremia and viral load in broncho-alveolar lavage fluid (BALF) were lower in the SU1-bel group. Infection with SU1-bel induced an enhanced adaptive immune response with greater interferon (IFN)-γ responses and an earlier PRRSV-specific antibody response. Infection with PRRSV did not affect the response to vaccination against ADV. Our results indicate that the increased clinical and pathological effect of the SU1-bel strain is more likely to be caused by an enhanced inflammatory immune response rather than higher levels of virus replication.

Further, we characterized the *in vitro* and *in vivo* response of the two European subtype 1 strains, Belgium A and Lelystad-Ter Huurne (LV), and the virulent subtype 3 strain, Lena, in bone marrow-derived dendritic cells (BM-DC) (*in vitro*) and alveolar macrophages (*in vitro* and *in vivo*). Infection with the Lena strain resulted in a higher level of apoptosis of cells *in vitro* and a higher level of infectivity *in vitro* and *in vivo* than the other virus strains. Furthermore, infection with Lena resulted in a small downregulation of the immunologically relevant cell surface molecules SLA-I, SLA-II and CD80/86 *in vitro*, and SLA-II *in vivo*. In spite of these differences, *in vitro* cytokine responses did not differ significantly between strains, except for the absence of IL-10 production by Lena in BM-DC. The higher infectivity, apoptosis and downregulation of the cell surface molecules, may have contributed to the increased pathogenicity of Lena, and have dampened virus specific immune responses, but contributed to a high systemic inflammatory reaction. These characteristics of this virus strain could explain the delayed and decreased adaptive immune responses observed after infections with this strain (Weesendorp et al., 2013, Vet Microb).

Also, a new polarized nasal mucosa explant system was used to study the invasion of the low virulent subtype 1 PRRSV strain Lelystad (LV) and the highly virulent subtype 3 PRRSV strain Lena at the portal of entry (Frydas et al. 2013, Vet Res). Different cell types of the monocytic lineage (alveolar macrophages (PAM), cultured blood monocytes and monocyte-derived dendritic cells (moDC)) were enclosed to examine replication kinetics of both strains in their putative target cells. At 0, 12, 24, 48 and 72 hours post inoculation (hpi), virus production was analyzed and the infected cells were quantified and identified. Lena replicated much more efficiently than LV in the nasal mucosa explants and to a lesser extent in PAM. Differences in replication were not found in monocytes and moDC. Confocal microscopy demonstrated that for LV, almost all viral antigen positive cells were CD163+Sialoadhesin (Sn)+, which were mainly located in the lamina propria of the respiratory mucosa. In Lena-infected nasal mucosa, CD163+Sn+, CD163+Sn- and to a lesser extent CD163-Sn- monocytic subtypes were involved in infection. CD163+Sn- cells were mostly located within or in the proximity of the epithelium. Our results show that, whereas LV replicates in a restricted subpopulation of CD163+Sn+ monocytic cells in the upper respiratory tract, Lena hijacks a broader range of subpopulations to spread within the mucosa. Replication in CD163+Sn- cells suggests that an alternative entry receptor may contribute to the wider tropism of Lena.

**Task 4.2. Reverse genetic analyses using infectious cDNA clones to define the genetic basis for virulence of PRRSV**

Atypical porcine reproductive and respiratory syndrome (PRRS), which is caused by the Chinese highly pathogenic PRRS virus (HP-PRRSV), has resulted in large economic loss to the swine industry since its outbreak in 2006. However, to date, the region(s) within the viral genome that are related to the fatal virulence of HP-PRRSV remain unknown. In the present study, we generated a series of full-length infectious cDNA clones with swapped coding regions between the highly pathogenic RvJXwn and low pathogenic RvHB-1/3.9. Next, the *in vitro* and *in vivo* replication and pathogenicity for piglets of the rescued chimeric viruses were systematically analyzed and compared with their backbone viruses. First, we swapped the regions including the 5'UTR+ORF1a, ORF1b, and structural proteins (SPs)-coding region between the two viruses and demonstrated that the nonstructural protein-coding region, ORF1b, is directly related to the fatal virulence and increased replication efficiency of HP-PRRSV both *in vitro* and
in vivo. Furthermore, we substituted the nonstructural protein (Nsp) 9-, Nsp10-, Nsp11- and Nsp12-coding regions separately; or Nsp9- and Nsp10-coding regions together; or Nsp9-, Nsp10- and Nsp11-coding regions simultaneously between the two viruses. Our results indicated that the HP-PRRSV Nsp9- and Nsp10-coding regions together are closely related to the replication efficiency in vitro and in vivo and are related to the increased pathogenicity and fatal virulence for piglets. **Our findings suggest that Nsp9 and Nsp10 together contribute to the fatal virulence of HP-PRRSV emerging in China,** helping to elucidate the pathogenesis of this virus (Li et al., 2014, PLoS Pathog).

Task 4.3. Comparison of gene expression in alveolar macrophages and dendritic cells, infected in vitro with high and low virulence PRRSV strains

We investigated the transcriptome of PAMs in vitro at 12 h post-infection with two European PRRSV strains characterized by low (Lelystad, LV) and high (Lena) virulence through RNA-Seq (Badaoui et al., 2014, PlosOne). We found that the expression levels of genes, isoforms, alternative transcription start sites (TSS) and differential promoter usage manifested a complex pattern of transcriptional and post-transcriptional gene regulation upon infection with the two strains. Gene ontology analysis confirmed that infection of PAMs with both the Lena and LV strains affected signaling pathways directly linked to the innate immune response, including interferon regulatory factors (IRF), RIG1-like receptors, TLRs and PKR pathways. **The results confirmed that interferon signalling is crucial for transcriptional regulation during PAM infection. IFN-beta1 and IFN-alpha-omega, but not IFN-alpha, were up-regulated following infection with either the LV or Lena strain.** The down-regulation of canonical pathways, such as the interplay between the innate and adaptive immune responses, cell death and TLR3/TLR7 signalling, was observed for both strains, but Lena triggered a stronger down-regulation than LV. This analysis contributes to a better understanding of the interactions between PRRSV and PAMs and outlines the differences in the responses of PAMs to strains with different levels of virulence, which may lead to the development of new PRRSV control strategies.

In addition, we performed many bioinformatics studies to deepen our understanding of the pig immune response to PRRSV infection. Hence, i) we used publically available microarrays data to perform meta-analysis studies (Badaoui et al., 2013, BMC Genomics) and ii) we participated into the structural and functional annotation of the pig immunome (Dawson et al., 2013, BMC genomics).

Task 4.4. Identification of viral ligands and protease cleavage sites

The identification of the M/GP3 glycoprotein complex of PRRSV as a ligand for sialoadhesin (Van Breedam et al., 2010, PLoS Pathog) and of the GP2 and GP4 proteins as a ligand for CD163 (Das et al., 2010, J Virol) allowed us to **design a model of PRRSV entry into the porcine macrophage** (Van Breedam et al., 2010, J Gen Virol). Initially, the PRRSV virion attaches to heparan sulphate GAGs present on the macrophage surface (a). Subsequently, the virus binds to the sialoadhesin receptor via M/GP5 glycoprotein complexes present in the viral envelope (b). Upon attachment to sialoadhesin, the virus–receptor complex is internalized via a process of clathrin-mediated endocytosis (c). Upon internalization, the viral genome is released from the early endosome into the cytoplasm of the host cell (d), thereby initiating the transcriptional and translational events required for the formation of new virions. Scavenger receptor CD163 is essential for this genome release and may exert its function through interaction with GP2 and GP4. In addition, cellular proteases have been implicated and also a modest pH drop within the early endosome is crucial for viral genome release.

WP5 Immunobiology of PRRS, Leader: Dr. Falko Steinbach, AHVLA (United Kingdom)

Task 5.1. Effects of PRRSV infection upon different types of immune cells

Immune cells of the monocyte-macrophage and the dendritic cell (DC) lineages carry out several distinct functions that link the innate to the adaptive immune system. Viruses that target these cells therefore cause a particular challenge to the generation of an immune response upon infection and the production of vaccines. PRRSV falls into this category of viruses, which formed the rational for this particular task.
We firstly aimed to analyse the baseline of PRRSV infection using porcine alveolar macrophages (PAM) as susceptible and monocytes as refractory antipodes in the interaction with PRRSV. Not surprisingly perhaps the infection of PRRSV in PAM was variable, to the extent of being poor in some cases. To our surprise though peripheral blood monocytes (PBM) were not completely refractory and spontaneously became very susceptible within 48 hours. We therefore analysed this further not least since PBM are much easier to obtain. Firstly, the effect of the cytokines M-CSF and GM-CSF, known to enhance viability of PBM was analysed on the replication of PRRSV. Interestingly, it was demonstrated that M-CSF is able to stimulate proliferation in porcine monocytes. Neither factor, however enhanced the replication of PRRSV in monocytes and therefore another series of cytokines and immune-modulators such as LPS or corticosteroids was analysed and indeed some which enhanced the expression of receptors for PRRSV also enhanced the replication of PRRSV, but some details as to the mode of action require further analysis.

Studies on the interaction of PRRSV with porcine macrophages were carried out with the aim being to determine the impact of IFN-γ (M1-polarisation), IL-4 (M2 polarisation) and IFN-beta activation of macrophages on the susceptibility to genotype 1 and 2 PRRSV strains varying in virulence. Results showed that undifferentiated and M2 macrophages were highly susceptible to all PRRSV isolates. In contrast, M1 and IFN-beta -activated macrophages were resistant to low pathogenic genotype 1 PRRSV but not or only partially to genotype 2 PRRSV strains. Interestingly, highly virulent PRRSV isolates of both genotypes showed particularly high levels of infection in both M1 and IFN-beta-treated macrophages. These results indicate that by using IFN-γ and IFN-beta stimulated macrophages it is possible to discriminate between PRRSV varying in genotype and virulence. Genotype 2 PRRSV strains are more efficient at escaping the intrinsic antiviral effects induced by type I and II IFNs. This in vitro model will help to identify viral genetic elements responsible for virulence, and provide information important in the understanding of PRRS pathogenesis and vaccine design. The results also suggest that monocyte-derived macrophages can be used as a PRRSV infection model instead of alveolar macrophages, reducing the number of pigs required for the collection of cells (Garcia-Nicolas et al., 2014, Virus Res.).

Studies on the interaction of PRRSV comparing various stages of myeloid cells, including monocytes, macrophage and myeloid dendritic cells (DC) showed that all are susceptible to PRRSV infection with monocyte-derived DC (MoDC) being the least susceptible population. While all myeloid populations could be infected with PRRSV, there were significant differences in the kinetics of replication between the cell types. This may reflect the poor expression of the prototype PRRSV receptors CD169 and CD163, which are largely absent on MoDC, thus making it necessary for PRRSV to utilise other pathways for entry.

Regarding the stimulation of an immune response, it was demonstrated that PRRSV can induce potent IFN-alpha responses in plasmacytoid dendritic cells. These are the most potent producers of type I IFN in response to viruses and are hence important not only to the innate immune defence. Through their localization in lymphoid tissue the released IFN also plays a major role in promoting adaptive immune responses and consequently, the ability of PRRSV to activate pDC was considered of importance. Our findings demonstrate that pDC respond to PRRSV and suggest that suppressive activities on pDC, if any, are moderate and strain-dependent. Thus, pDC may be a source of systemic IFN-α responses reported in PRRSV-infected animals, further contributing to the puzzling immunopathogenesis of PRRS (Baumann et al., 2013, Vet Res).

Toll-like receptors are important sensors of infection and their signaling initiates immune reaction. The effects of PRRSV infection upon the expression of endocytic TLR was investigated using porcine alveolar macrophages (PAM) infected with different PRRSV isolates (3262, 3267 and DV). Cells were collected to determine the kinetics of viral replication in relation to the expression of TLR3, 7 and 9. Infection with PRRSV did not affect relative levels of any TLR mRNA transcript however resulted in
differences in the proportion of TLR3\(^+\) cells. The infection with isolate 3262 resulted in an increase of TLR3\(^+\) cells. In contrast for isolate 3267 a lower proportion of TLR3\(^+\) PAM was observed. Isolate 3262 replicates to lower levels than 3267. Apparently, TLR 7 and 9 were not affected apparently by PRRSV infection. These observations suggest that at least TLR3, a sensor of double-stranded RNA as it occurs during PRRSV replication, is regulated differentially by different genotype 1 PRRSV isolates and this seems to be related to the replication levels of each isolate. Since mRNA transcripts were kept constant this regulation probably occurs at a post-transcriptional level (Kuzemtseva et al., 2014, Vet Immunol. Immunopathol.).

Finally, if PRRSV replicates in myeloid cells including DC, a danger is the killing of these immune-modulatory cells. The development of apoptosis in DC caused by PRRSV was assessed using bone-marrow DC infected with different genotype 1 PRRSV isolates. The results indicated that with isolates which replicate to a low level only, the induction of apoptosis after 24 h is very low. In contrast for isolates with higher replication levels, apoptosis, however, is observed in both infected and bystander cells. The results indicate that PRRSV infection may be able to cause damage well beyond the infected cells.

**Task 5.2. Assessment of the importance of regulatory immunity in the course of PRRSV infection**

Next to the infection of immune cells, a second option to interfere with the generation of an efficient immune response is the formation of regulatory immunity. Regulatory immunity represents a technical term used to describe variants of regulatory T cells (Treg). These Treg cells, which are important in the context of allergy and auto-immunity have recently been described extensively also in the context of infection immunity, where they are detrimental. While the particular events surrounding the generation of Tregs remain enigmatic, certain cytokines such as IL-10 and TGF-beta seem to play a particular role. Certain strains of PRRSV have been shown to induce such cytokines, which formed the rational for studying these cells in this project.

However, making use of the in vivo experiments in WP4 and Task 5.3, no abnormal levels of Tregs or evidence for the induction of Treg after primary infection was detected with neither of the strains used. Thus it may be concluded that Treg seem to play a negligible role in the pathogenesis of PRRSV-1 infection. Also, IL-10-inducing capabilities did not correlate with longer viraemia or weaker cell-mediated response. However, it was also demonstrated that different outcomes of the infection may be, at least partially, related to the immunological properties of the PRRSV strain used for the infection. One strain (3267) that did not induce significant release of TNF-alpha and IL-10 produced a more persistent infection, with low levels of cell-mediated response and higher levels of antibodies. Also levels of IL-8 and TGF-beta differed depending on the strain used for primary inoculation (Diaz et al., 2012, Vet Res).

**Task 5.3. Identification of T cell epitopes relevant to protection**

Previous studies had shown that virus inhibiting, neutralising antibodies are only detected late (if at all) upon PRRSV infection. Thus it is evident that when PRRSV infections are cleared this is mainly due to the so called cellular immunity, which in its adaptive form is best represented by cytotoxic T cells, which may require the support of T helper cells for their efficient formation. Previous to this study, little was known about the targets of T cells upon PRRSV infection, which is confounded by the high variability across PRRSV strains. We therefore sought to address this issue systematically within PoRRSCon.

Therefore, a synthetic peptide library was designed to identify T cell epitopes across the proteome of PRRSV. Using the sequence data from the EU PRRSV reference strains Lelystad and Olot91, synthetic overlapping 16mer peptides were obtained, corresponding to the 12 non-structural proteins and seven structural proteins. PBMC from all animals showed significant IFN-\(\gamma\) responses following in vitro stimulation with PRRSV and peptides pooled to represent PRRS proteins. From the screening of these pools, putative antigenic peptides were identified in NSP1, NSP2, NSP5, RdRp, GP5 and M proteins. The
screening of T cells from infected pigs showed again significant responses against NSP1b and NSP2 and M. Interestingly, all the SU1-bel inoculated animals mounted the greatest response against peptides representing NSP5 of Olot91, suggesting that this protein contained highly conserved T cell epitopes. Further characterisation of the M and NSP5-specific CD8 T cells showed them to possess a phenotype resembling a mixed population of effector and effector memory T cells. Significantly, these cells appear to have a cytotoxic function and direct lytic activity against NSP5 peptide pulsed cells.

WP6 Inactivated/subunit marker vaccines, Leader: Dr. Hans Nauwynck, UGent (Belgium)

Task 6.1. Adaptable inactivated vaccine against European type PRRSV strains (subtype 1) that escape from vaccination immunity,

The aim of this task was to test the efficacy of inactivated vaccines developed according to an innovative procedure in the laboratory of partner 1 (patent application EP2257808, February 29, 2008; Vanhee et al., 2009).

(i) European type PRRSV strains (subtype 1) that escape from vaccination immunity:

We demonstrated that experimental inactivated vaccines based on two recent field isolates (07V063 and 08V194, isolated from pigs after a PRRSV outbreak in herds vaccinated with a European-type, attenuated vaccine (Porcilis® PRRS)) of European type PRRSV (subtype 1), significantly shortened viremia in piglets upon homologous challenge (Geldhof et al., 2012, BMC Vet Res). Furthermore, the novel experimental inactivated PRRSV vaccine (07V063) primed the VN antibody response and slightly reduced the duration of viremia in gilts. It also reduced the number of virus-positive fetuses and improved the fetal survival, but was not able to fully prevent congenital PRRSV infection. The reduction of fetal infection and pathology is most probably attributable to the vaccine mediated decrease of PRRSV transfer from the endometrium to the fetal placenta. Screening of the endometrium/fetal placenta for virus replication and virus-induced changes proved to be a useful tool to study the pathogenesis of congenital PRRSV infection and validate the PRRSV vaccine performance. Hence, in this study, an advanced protocol was proposed to test the PRRSV vaccine efficacy. Finally, requirements were summarized for primary testing of PRRSV vaccines in pregnant animals which may be helpful in development of a monograph (Karniychuk et al., 2012, Theriogenology).

We investigated the capacity of commercially available attenuated/inactivated PRRSV vaccines and autogenous inactivated PRRSV vaccines developed in his laboratory to boost the antibody immunity against currently circulating PRRSV variants in PRRSV-immune sows, i.e. sows that have a pre-existing PRRSV-specific immunity due to previous infection with or vaccination against the virus. The results of this study indicate that inactivated farm-specific PRRSV vaccines and commercial attenuated vaccines can be useful tools to boost PRRSV-specific humoral immunity in sows and reduce viremia in weaned piglets (Geldhof et al., 2013, Vet Microbiol).

These studies consolidate the knowledge that the efficacy of the current PRRSV vaccines is highly depending on the circulating virus strain. The observation that homologous BEI-inactivated vaccines can provide a predictable degree of protection against a specific virus variant suggests that such vaccines may prove useful in case virus variants emerge that escape the immunity induced by the attenuated vaccines. A farm-specific vaccine can induce VN antibodies and offer partial protection upon homologous challenge and therefore may be used in addition to the commercially available vaccines on farms with PRRSV-related problems. Valorization of this new type of European (subtype 1) PRRSV inactivated vaccine that is safe, efficacious, and adaptable, that offers partial protection against viremia and reproductive failure, as well as protective colostral immunity, has been achieved.

(ii) virulent European type PRRSV strains from Eastern Europe (subtype 3)

Infection of pigs with the East European subtype 3 PRRSV strain Lena (WP3, Karniychuk et al., 2010, BMC Veterinary Research) or SU1-bel is characterized by increased clinical signs and pathological
effects that could be possibly explained by an increased inflammatory immune response (see WP4 Task 4.1 and Morgan et al., 2013, Vet Microbiol).

Because available commercial vaccines have not been tested yet against the virulent strains from Eastern Europe, we evaluated the efficacy of a commercial attenuated European subtype 1 PRRSV vaccine upon challenge with the East European subtype 3 PRRSV strain Lena (83.3% nucleotide identity with the vaccine DV strain, Porcilis®PRRS, Intervet BV). This study showed that vaccination of pigs with an attenuated European subtype 1 vaccine provides a partial protection against a subsequent exposure to the highly pathogenic East European subtype 3 PRRSV strain Lena (Trus et al., 2014, Vaccine).

In addition, based on the knowledge generated by the development of inactivated vaccines for PRRSV, a general review on virus inactivation was published by partner 1. The aim of this review is to make researchers aware of the benefits of an efficient quality control system for prediction of a developed vaccine's efficacy. Two major goals should be addressed when inactivating a virus for vaccine purposes: first, the infectious virus should be inactivated completely in order to be safe, and second, the viral epitopes important for the induction of protective immunity should be conserved after inactivation in order to have an antigen of high quality. Therefore, some problems associated with the virus inactivation process, such as virus aggregate formation, protein crosslinking, protein denaturation and degradation should be addressed before testing an inactivated vaccine in vivo (Delrue et al., 2012, Expert Rev Vaccines).

Task 6.2. Development of subunit vaccines using the baculovirus system

During the project, three approaches to obtain subunit vaccines have been carried out. The initial one was based on GP5/M proteins. The results and the information that arose during the development of the project directed the efforts towards Gp3. The last approach was focused on the co-expression of several PRRS structural proteins.

For several years, GP5 has been the target for PRRSV vaccine development because one of the most important potential neutralizing epitopes was described in its ectodomain. Therefore, this part of the sequence was expressed, together with two different epitopes from the Matrix (M or ORF6 protein). These epitopes were selected based on their role in the GP5/M complex formation (M1) or in their potential involvement in the B and T cell responses. In order to preserve the GP5 ectodomain structure, the sequence was expressed fused with the two M epitopes (EctoM1 or EctoM4 recombinant proteins). The two proteins were expressed in the baculovirus system. After purification, the proteins were used to immunize mice and, subsequently, to obtain monoclonal antibodies (mAbs). The seroneutralization assays performed with mice sera and mAbs indicated that the recombinant proteins were not able to elicit neutralizing Abs. This result, together with data obtained with other vaccine types focused on GP5/M proteins, did not justify pig immunization. Nevertheless, a panel of mAbs that mainly recognized M protein was obtained.

The study of linear antigenic regions by UGent pointed out one potential neutralizing B cell epitope in the GP3 ectodomain (Vanhee et al., 2011, Vaccine). Based on this information, three different constructions of GP3 ectodomain were made and expressed in the baculovirus system. One of them, GP3.14, which contained the described epitope and lacked the GP3 signal sequence and the trans-membrane region, was purified and produced in sufficient amounts to perform vaccination-challenge experiments. The analysis of the sera indicated that the recombinant GP3.14 was highly immunogenic and generated Abs before the challenge measured by a GP3 fragment-based ELISA (challenge was performed at day 66 post vaccination, pv). Further analysis indicated statistical differences between vaccinated animals and the control group from day 38 pv (p<0,0001). An indirect ELISA assay where PRRS virus was used as coating antigen indicated that these Abs were able to recognize the virus before the challenge. Statistical differences were significant from day 38 pv until day 7 post challenge (p<0,0001-0,005). After that day, there were no significant differences between the Abs generated against the virus by the two groups of

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pigs: this could be due to the contribution of Abs anti N protein developed in both groups of animals. In addition, there were no significant differences between vaccinates and controls neither in the levels of two Acute Phase Proteins (as an indirect determination of the innate immune response) nor in the virus titers in sera. Seroneutralization experiments were performed with pools of sera at days 0 and 66 pv (day of the challenge) and at day 21 post-challenge. The results indicated that the Abs generated after the GP3.14 immunization were not neutralizing, supporting what was observed after the challenge in terms of viraemia and clinical symptoms. As mentioned in the previous sections, the reasons for the lack of protection could be differences between the structure of the GP3 epitopes presented after virus infection and after vaccination with the recombinant protein and, also, the contribution of different epitopes in other PRRSV proteins to the global protective response.

To check the latter possibility and in parallel with the experiments performed with vector vaccines, the co-expression of several structural proteins of the virus to try to produce PRRS VLPs was planned. Nam et al. (2013, Arch Virology) described the formation of PRRS basic structures or VLPs by the co-expression of GP5 and M proteins in the baculovirus system. Based on these results, the idea was to incorporate to these VLPs more structural proteins that could contribute to the immune response after vaccination. Six structural proteins of PRRS were cloned in two plasmids: GP5 and M in one plasmid; GP2, E, GP3 and GP4 in another plasmid. By the end of the project timeframe, GP5 and M co-expression was detected in insect cell cultures. It remained to be determined if these proteins are able to form the basic VLP in order to perform co-infections that allow the addition of the remaining proteins.

**WP7 Recombinant live marker vaccines, Leader: Dr. Luis Enjuanes, CSIC (Spain)**

Current modified live vaccines against PRRSV lead to much better protection than other vaccine types. Nevertheless, they have several problems such as incomplete protection against heterologous strains, vaccine virus shedding and spread, and possible reversion to virulence. Therefore vector-based vaccines could represent an advantage to stimulate both humoral and cellular immune responses against PRRSV. In WP7 the use of two different vectored vaccines was proposed: (1) recombinant transmissible gastroenteritis virus (TGEV) derived vectors, and (2) replicon vectors based on classical swine fever virus (CSFV) or vesicular stomatitis virus (VSV). In both cases, several PRRSV antigenic structures, based on GP5-M heterodimer or GP2-GP3-GP4 heterotrimer were explored.

**Development of vaccines based on rTGEV vectors, expressing GP5/M or GP2a/GP3/GP4**

**Expression of GP5 fragment and M protein.** A set of rTGEV vectors co-expressing different GP5 domains and M protein were obtained. When compared with full-length GP5 expression (Cruz et al., 2010, Virus Res), stability was significantly increased by the expression of GP5 ectodomain. Unfortunately, after 16 passages in tissue culture, the stability of this vector was reduced. Therefore, to further eliminate GP5 toxic regions, a smaller GP5 fragment was cloned into rTGEV vector. This fragment contained part of the GP5 ectodomain, including antigenic domains involved in protection, and lacking the signal peptide

Recombinant TGEV vector co-expressing GP5 fragment and M protein was obtained with high titer. The stability of this vector was greatly improved when compared with full-length or GP5-ecto expression, as 70 % of the viral clones expressed the PRRSV antigen after 16 passages in tissue culture.

**Expression of other PRRSV envelope proteins.** Expression of full-length PRRSV GP3 protein, alone or in combination with other PRRSV proteins, was not achieved as this protein was highly toxic when expressed by rTGEV. Therefore, an rTGEV vector was generated, expressing a small GP3 domain containing the epitope recognized by neutralizing antibodies (according to the information obtained by UGent). A 100 % of the isolated rTGEV viral clones expressed GP3 fragment indicating that this GP3 domain was fully stable when cloned in rTGEV vector. This result was a significant improvement over previous attempts to express full-length GP3 protein.

An rTGEV virus expressing full-length GP4 protein was previously generated. This virus expressed high levels of GP4 in 90% of the infected cells. Nevertheless, GP4 expression was significantly reduced with
serial passages in tissue culture, due to the loss of the GP4 gene from the rTGEV genome. Therefore, similarly to the strategy used for GP3 protein, a small GP4 domain was cloned in rTGEV vector. This GP4 fragment contained the epitope recognized by neutralizing antibodies (according to the information obtained by UGent). The analysis of virus stability showed that 100% of the isolated viral clones expressed GP4 fragment indicating that this GP4 domain was fully stable when cloned in rTGEV vector.

**Expression of chimeric M proteins exposing PRRSV GP3 antigens.**

PRRSV M protein was fully stable when expressed by rTGEV vector. Therefore, in principle, it could be used as a scaffold to generate new antigenic structures. As M protein is involved in cellular immune response, and M protein T cell epitopes are highly conserved, the new antigenic structures could provide both homologous and heterologous cellular immune responses. In addition, theoretically, this approach would allow the design of cross-protective constructs by the expression of multiple linked antigens. Two locations in the M protein were found suitable for the insertion of small antigenic domains: the N-terminus and one internal loop exposed in the virus surface. As a first approach, a small GP3 fragment, containing the motif recognized by neutralizing antibodies, was inserted into these two M protein positions generating GP3fr-NtermM and GP3fr-Mloop fusion proteins, respectively.

rTGEV viruses expressing chimeric proteins were recovered and passaged 16 times in tissue culture. An RT-PCR analysis of the stability of the rTGEV vectors indicated that 90% of the isolated clones contained GP3fr-NtermM, and 100% of the clones contained GP3fr-Mloop. Fusion protein expression levels and stability were also evaluated by immunofluorescence analysis. Around 92% of the infected cells expressed GP3fr-NtermM, perfectly correlating with RT-PCR results. Expression of GP3fr-Mloop was detected in 73% of the infected cells, suggesting that in the native structure the FLAG epitope was most likely hidden and not accessible to the antibody used for the immunofluorescence.

The rTGEV-based vectors that fulfill stability criteria were selected for in vivo evaluation. Vaccinated and challenged animals were partially protected, as respiratory clinical symptoms, lung damage, and accumulation of pro-inflammatory cytokines such as IL-8 were lower in these animals compared with the non-vaccinated ones. In addition, a slight PRRSV viremia decrease was also observed in these animals at late times post-challenge. A fast and recall response against GP5 was observed in vaccinated animals after challenge. These animals elicited a lower level of neutralizing antibodies compared with the non-vaccinated ones, and higher levels of IFN-alpha at early times post-challenge. Altogether, our results suggested that both humoral and cell responses contributed to the decreased PRRSV infection in vaccinated animals, although the correlates of protection were not determined (Becares et al., 2014, Virology).

In summary, our results show that reduction of the heterologous gene size inserted in the TGEV-derived vector is a promising strategy to achieve stable expression. Additionally, as PRRSV M protein was stable in rTGEV, several antigenic structures were engineered using this protein as scaffold for the expression of small antigenic domains, resulting in high stability. The data indicated that rTGEVs expressing PRRSV antigenic structures of reduced size considerably increased their stability. Furthermore, immunization of piglets with live attenuated rTGEV vectors partially protected against PRRSV, with reduction of clinical signs and lung damage as well as a faster viremia decrease.

**Improvement of rTGEV vectors by decreasing their recombination rate.**

Although rTGEV is a promising vector, several heterologous genes were lost after few passages in tissue culture when inserted in the TGEV genome. We hypothesized that the analysis of viral proteins involved in RNA recombination would help to design improved rTGEV vectors stably expressing heterologous antigens. The analysis of rTGEV proteins modulating recombination rate was not included in the project proposal. The obtained results indicated that the best protection would be achieved by the expression of full-length PRRSV proteins. Therefore, rTGEV vectors should be improved by decreasing their recombination rate, allowing the expression of whole heterodimeric (GP5-M) or heterotrimeric (GP2-GP3-GP4) PRRSV protein structures.
Development of CSFV and VSV VRP-derived vaccines (replicon vectors) expressing GP5/M or GP2a/GP3/GP4

**CSFV and VSV replicons as vaccine vectors.**

RNA replicon vectors are viral genomes lacking one or more viral structural protein genes, which prevents production of progeny virus. CSFV and VSV replicons can be packaged in virus replicon particles (VRP) using complementing cell lines expressing the missing structural proteins. Previous work from P6 FDHA-IVI showed that VRP from CSFV replicons (CSFV VRP) can induce a protective immunity against CSFV (Suter et al, 2011, Vaccine) and can express influenza A virus (IAV) proteins, inducing a strong antibody response against IAV in rabbits (submitted for publication). VRP from VSV replicons (VSV VRP) were also used successfully to induce protection against IAV infections in chickens (Kalhoro, 2009, Vaccine; Halbherr et al, 2013, PLoS One) and bluetongue virus in sheep (submitted for publication).

**Expression of the PRRSV structural proteins with CSFV and VSV replicons.**

In the line of the previous results described above, a total of 16 VSV and 6 CSFV replicon constructs carrying the different PRRSV structural proteins, alone or in combination, were constructed in the present project. The VSV replicon could accommodate up to 3 adjacent full-length PRRSV gene cassettes, allowing expression of either GP5 and M together or GP2, GP3 and GP4 together from a single vector, respectively. In the CSFV replicons, only single gene insertions were evaluated. In some constructs, antigenic epitope tags (Flag, HA, and myc) were used to facilitate analysis of the proteins expressed. With the aim of enhancing both B-cell and T-cell responses against the PRRSV proteins through a “genetic adjuvant” effect, three different scaffold proteins were evaluated, using the PRRSV nucleoprotein N as a model. The N gene was cloned into the VSV replicon, in frame with the heat shock protein 70 (HSP70), a chaperon important in protein folding and transport, or with the high mobility group protein 1 (HMBG1), a pro-inflammatory protein released by necrotic cells, or with the non-structural protein 1 of bluetongue virus 8 (NS1BTV8), for presentation of multiple antigen copies on its tubular structures. N was also inserted in the CSFV replicon carrying a modified Npro gene in order to trigger the innate immune response by inducing type I interferon. With VSV VRP, all PRRSV glycoproteins and truncated variants thereof were expressed at high levels. With CSFV VRP, only very low levels of the N protein and of the GP3 ectodomain were detectable in cell culture.

VSV replicons were preferred to CSFV replicons as candidate PRRSV vector vaccines. Based on their high PRRSV protein expression levels, the VSV replicon constructs were preferred to the CSFV replicons for pre-evaluation of immunogenicity in mice and chicken as surrogate animal models. Selected VSV VRPs were subsequently evaluated as vaccine candidates in pigs. None of them were protective. Nevertheless, enhanced seroconversion after challenge with PRRSV suggested a VSV VRP-induced priming effect with T-helper-cell memory responses in pigs.

**WP8. Attenuated live marker vaccines obtained by reverse genetics and allowing DIVA, Leader: Dr. Fei Gao, SHVRI-CAAS (China)**

We have developed full-length cDNA clones of the highly pathogenic Chinese strains (genotype 2) and genotype 1 strains that enable to introduce mutations that potentially attenuate the virus and to introduce a marker in an existing attenuated vaccine. Using reverse genetics, we studied which (part of) viral genes are essential for infectivity and focused the research on identifying possible markers that would allow differentiation of animals vaccinated or infected (DIVA).

- Deletion mutant based on Nsp2 coding region: The Chinese HP PRRSV JX143 was attenuated via MARC-145 cell passage for 100 times, from which a plaque-purified virus, designated as JXM100, was characterized. JXM100 grew to higher titers, showed faster growth kinetics and larger plaque sizes than JX143. For JXM100 in vivo testing model, twenty-four 28-day-old PRRSV-free piglets were obtained and divided randomly into four groups, i.e., six piglets in each group. Each treatment group was hosed individually. Piglets from the JXM100-infected group and the piglets in the control group did not exhibit
high fever or other clinical signs at 0–28 dpc. Results demonstrated that JXM100 was attenuated in piglets and that it could protect them from lethal infection with HP PRRSV JX143. Sequence analysis demonstrated the presence of a continuous 264 nucleotide (88 amino acids; 88 aa) deletion in the nsp2 region and 75 random nucleotide mutations throughout the genome. This 88 aa sequence was cloned, expressed in E.coli, purified and used to generate polyclonal antibody and as recombinant antigen for ELISA. ELISA results demonstrated that del88 aa was indeed immunogenic, and convalescent serum contained antibodies directed against this epitope. Pigs inoculated with JXM100 with an 88 aa deletion (del88) in nsp2 elicited a strong antibody response against the N protein but they did not develop antibody against the del88, whereas strong reactivity was observed in sera obtained from piglets infected with JX143 using the same del88-based ELISA. We confirmed that the 88 aa mutation in the nsp2 was stably maintained in the replicating virus. Accordingly, JXM100 might be a candidate vaccine to protect against HP-PRRSV and del88 can be used for DIVA as a genetic marker for serologically differentiating JXM100 from JX143 (Wang et al., 2013, Virus Res).

1) An infectious cDNA clone designated pAJXM, constructed from the attenuated genotype 2 JXM100 virus, served as a backbone for:
* Constructing GP5 glycosylation site mutants for studying the importance of N-linked glycan addition at certain sites in GP5 for production of infectious viruses and viral infectivity. No viremia and antibody response were detected in piglets that were injected with a mutant without all N-linked glycans in GP5. These results suggest that the N-linked glycosylation of GP5 is critically important for virus replication in vivo. The study also showed that removal of N44-linked glycan from GP5 increased the sensitivity of mutant virus to convalescent-phase serum samples but did not elicit a high-level neutralizing antibody response to wt PRRSV. The results obtained from the present study have made significant contributions to better understanding the importance of glycosylation of GP5 in the biology of PRRSV (Wei Z et al., 2012, J Virol).

* Studying the importance of ORF5a for virus infectivity. We found that RNA changes in the overlapping region (1–104 nucleotide, nt) between ORF5 and ORF5a introduced by codon-optimized GP5 was lethal for virus viability, suggesting that the nt changes or amino acid (aa) mutations in the GP5 or ORF5a protein did not allow the production of infectious virus. Furthermore, inactivation of ORF5a expression in the context of type 1 (pSHE) and type 2 (pAJXM and pAPRSS) full-length PRRSV cDNA clones was lethal for the production of infectious virus, while viable PRRSV could be recovered by expressing ORF5a protein in trans, suggesting that ORF5a protein was essential for virus viability. Finally, ORF5a protein could be putatively extended to 63 amino acids by inactivation of the downstream stop codon candidates, thereby demonstrating that the C-terminus of ORF5a may be variable (Sun L. et al., 2013, Virus Res).

2) An infectious cDNA clone pSHE (genotype 1) virus was developed. The genotype 1 PRRSV strain, vSHE, was rescued from the infectious clone pSHE (GQ461593; Yuan et al., unpublished data), based on the attenuated vaccine strain AMERVAC-PRRS/A3 (Hipra Laboratory). The vSHE shares 95% identity with LV strain (prototype strain of type 1 PRRSV) at the genome level. This infectious clone was used for:
* Studying the function of the PRRSV envelope proteins: We constructed viable chimeric viruses in which the envelope protein genes from ORF2a to ORF5 of vSHE (genotype 1) were swapped into the genetic backbone of vAPRSS (genotype 2, attenuated strain). The envelope proteins of genotype 1 were fully functional in genotype 2 PRRSV, and the rescued chimeric progeny viruses showed robust genetic stability and similar replication properties to the parental strains in vitro. To our knowledge, this is the first study to report the substitution of complete ORFs between different genotypes of porcine arterivirus. These findings pave the way to further elucidate the structure–function relationship of PRRSV envelope proteins, and may enable the development of novel marker vaccines that can be used to differentiate vaccinated from infected animals ((Tian et al., 2011, Virology)

* Studying the functionality of the 5′ untranslated region of PRRSV. The 5′ untranslated region (UTR) is believed to be essential for the replication of PRRSV, yet its functional mechanism remains largely unknown. In this study, to define the cis-acting elements for viral replication and infectivity, the 5′ UTR
swapping chimeric clones pTLV8 and pSHSP5 were constructed based on two different genotypes full-length infectious cDNA clone pAPRRS and pSHE backbones. Between them, vTLV8 could be rescued from pTLV8 and had similar virological properties to vAPRRS, including phenotypic characteristic and RNA synthesis level. However, pSHSP5 exhibited no evidence of infectivity. Taken together, the results presented here demonstrate that only the 5’ UTR of type 1 PRRSV did not affect the infectivity and replication of type 2 PRRSV in vitro. The 5’ UTR of type 2 PRRSV could be functionally replaced by its counterpart from type 1 (Gao et al., 2013, Virology).

* Studying the role played by disulfide linkages mediating nucleocapsid dimerization in Porcine Arterivirus infectivity. The nucleocapsid (N) proteins of genotype 1 and 2 of PRRSV share only approximately 60% genetic identity, and the functionality of N in both genotypes, especially its role in virion assembly, is still poorly understood. We demonstrated that the ORF7 3’ untranslated region or ORF7 of genotype 1 is functional in the genotype 2 PRRSV background. Based on these results, we postulated that the cysteine at position 90 (Cys90) of the genotype 2 N protein, which corresponds to an alanine in the genotype 1 protein, is nonessential for virus infectivity. The replacement of Cys90 with alanine confirmed this hypothesis. We then hypothesized that all of the cysteines in the N protein could be replaced by alanines. Mutational analysis revealed that, in contradiction to previously reported findings, the replacement of all of the cysteines, either singly or in combination, did not impair the growth of either genotypes 1 or 2 of PRRSV. Treatment with the alkylating agent N-ethylmaleimide inhibited cysteine-mediated N dimerization in living cells but not in released virions. Additionally, bimolecular fluorescence complementation assays revealed noncovalent interactions in living cells among the N and C termini and between the N-terminal and C-terminal regions of the N proteins of both genotypes of PRRSV. These results demonstrate that the disulfide linkages mediating the N dimerization are not required for PRRSV viability and help to promote our understanding of the mechanism underlying arterivirus particle assembly (Zhang et al., 2012, J Virol).

-Deletion marker in the Nucleocapsid protein: N protein is the most abundant and important structural protein in PRRSV virion, and plays a crucial role in virion assembly. In this study, we found that the N-terminal residues 5–13 and last four C-terminal residues were non-essential for genotype 2 PRRSV viability. In addition, we also demonstrated that deletion in the middle region of N protein did not block the production of infectious virus. Our study is believed to be the first report that the inter-genotypically variable N terminal and internal residues of N protein could tolerate deletion without affecting type 2 PRRSV viability in cultured cells, while discrete inter-genotypically conserved terminal residues play crucial roles in viral RNA synthesis and/or virus growth. The nonessential regions identified here could be further utilized as insertion site for foreign gene tag and the rescued viruses could be the candidates for genetic marker vaccine (Tan et al., 2011, Virus Res).

Indeed, by means of reverse genetics, a marker virus (v7APMa) was generated with a mutant N protein that differs from the wild-type strains (vAPRRS, genotype 2, attenuated strain). The N-terminal of N protein (amino acids 1–11 of ORF7) was deleted and replaced with the 7APMa (a 15 aa peptide) by means of reverse genetics. In order to explore the potential of the novel strategy in the N protein to use as a site for positive marker, the characteristics of rescued virus (v7APMa) were evaluated both in vitro and in vivo. v7APMa shows stable inheritance in cell culture and the virologic characteristics of the marker virus in vitro showed that v7APMa replicates well as its parental strain. In addition, a peptide-based ELISA was developed to detect the specific antibodies for the introduced 7APMa peptide. Four-week-old pigs free of PRRSV obtained from a PRRS free farm were randomly divided into three groups and housed in separate pens, three pigs for each group. Two groups were infected intramuscularly (2 ml) at day 0 with 10exp6 TCID50/ml of vAPRRS or v7APMa and another group was mock infected. The v7APMa marker virus induced a similar level of anti-N protein antibodies than the parental vAPRRS and robust antibodies against the marker peptide, from 14 days post infection and sustained for the 35 days of the study. In contrast, sera from the parental vAPRRS and mock infected animals gave negative results for the 7APMa peptide. This approach, using a rationally designed marker virus, provides a new potential mutant basis for further development of PRRSV novel vaccines.
Altogether, the results indicated that the peptide-based ELISA constitutes an appropriate DIVA for the v7APMa vaccine (Lin et al., 2012, Virus Gene).

-Other essential regions in the PRRSV genotype 2 genome:
  *The 3’ untranslated region (3' UTR), including the poly (A) tail, reportedly plays an important role in arterivirus replication, but the roles of the cis-acting elements present in the 3’ UTR of PRRSV remain largely unknown. In the present study, PCR-based mutagenic analysis was conducted on the 3’ UTR of PRRSV infectious full-length cDNA clone pAPRRS to investigate the structure and function of the conserved terminal nucleotides between the poly (A) tail and the 3’ UTR region. Our findings indicated that the conservation of the primary sequence of the 3’ terminal nucleotides, rather than the surrounding secondary structure, was vital for viral replication and infectivity. Four nucleotides (nt) (5’-(15517)AAUU(15520)-3’) at the 3’ proximal end of the 3’ UTR and the dinucleotide 5’-AU-3’ exerted an important regulatory effect on viral viability. Of the five 3’-terminal nucleotides of the 3’ UTR (5’-(15503)AACCA(15507)-3’), at least three, including the last dinucleotide (5’-CA-3’), were essential for maintaining viral infectivity. Taken together, the 3’-terminal conserved sequence plays a critical role in PRRSV replication and may function as a contact site for specific assembly of the replication complex (Yin et al., 2013, Arch Virol).*  

*Further, genetic manipulation of a transcription-regulating sequence of PRRSV revealed key nucleotides determining its activity. These results provide more insight into the molecular mechanism of genome expression and subgenomic mRNA transcription of PRRSV (Zheng et al., 2014, Arch Virol).*

**Description of the potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of results (10p. max.)**

1. Potential impact

PoRRSCon is giving a significant response to the call “Porcine reproductive and respiratory syndrome (PRRS): new generation, efficient and safe vaccine, new control strategies (KBBE-2009-1-3-01), launched in Theme 2 of Food, Agriculture and Fisheries and Biotechnology”: “Building a European Knowledge-Based Bio-Economy (KBBE)”, Area “Optimised animal health, production and welfare across agriculture, fisheries and aquaculture”

Indeed, the European and international added value of the project lies in mutual knowledge generation, enhanced collaboration between different scientific disciplines, and in an improve preparedness for eventual introduction of higher virulent strains. The project will contribute to reduce economic losses and hence increase competitiveness of EU pig producers, improve trade and provide added value along the production chain. The project is developing new safe and efficacious vaccines and is contributing to the design of new control strategies.

PoRRSCon brings together a group of European and Asian scientists that have a strong expertise in different disciplines: molecular epidemiology, virology, immunology, vaccine development, veterinary sciences and genomics. Bringing these experts together and linking them into different PRRS research lines guaranteed a strong output (see below). The additional knowledge on molecular epidemiology (PRRSV characterization on different continents), pathogenesis and virulence (e.g. the capacity of the virus to persist in the pig for weeks without being eliminated by the immunity, molecular PRRSV entry mechanism, virulence genes), immunobiology (e.g. especially the distorted immune response allowing the virus to persist and the immune modulation, allowing other pathogens/toxins to cause disease) generated by the project will facilitate the development of the necessary tools to control PRRSV. A new type of European (subtype 1) PRRSV inactivated vaccine that is safe, efficacious, and
adaptable, that offers partial protection against viremia and reproductive failure, as well as protective colostral immunity, has been developed and tested during the course of the project. This new generation of safe and efficacious vaccines is now in the valorization stage at the laboratory of the coordinator. This type of vaccines will directly fill the need of veterinarians and pig producers to combat enzootic infections with PRRSV. These vaccines are designed for easy adaptation, which is necessary when new virus variants appear that are no longer controlled by the immunity given by the former registered vaccines. Being able to give a quick response to new variants will reduce economic losses and hence increase the competitiveness of pig producers in general. These adaptable vaccines are extremely important in protecting a whole pig population against epidemics of newly emerging, highly virulent PRRSV strains. Because highly virulent strains appear unexpectedly, they cause real disasters in pigs. High morbidity and mortality are concerns of animal welfare, economics (production, import/export, free trade) and food supply for man. The latter is especially important in developing countries. The recent appearance of highly virulent PRRSV strains in certain regions of China and Vietnam and other parts of Asia, caused a 30 to 50% loss of the pig population and resulted in regional shortages of pig meat on the market.

During the project, marker attenuated vaccines and serological assays that allow differentiation between infected and vaccinated animals (DIVA principle) were developed and tested in vivo. We showed that occurrence of a spontaneous deletion (negative marker) following passages to obtain an attenuated strain or the introduction of a recombinant peptide (positive marker) by reverse genetics were two successful strategies that allowed DIVA. This knowledge will be useful when developing new generation attenuated vaccines that should also be safer with regards to reversion to virulence. Such marker vaccines and DIVA assays tools are essential for the eradication of enzootic PRRSV, a worldwide goal. Eradication of the virus will improve animal health, which is directly linked with a better animal welfare, improvement of pig production and increase of competitiveness of pig producers. Further, it will facilitate trade and reduce the use of antibiotics during porcine respiratory problems, because PRRSV is an important component in causing respiratory disease together with other pathogens/toxins. Reduction of the use of antibiotics will improve meat quality and food safety. Marker vaccines and DIVA assays are efficient tools to control diseases, like attested by the eradication of Aujeszky’s disease virus in Europe using the combination of gE-negative marker vaccines and gE DIVA assays. This approach is very cost efficient. Further, it allows, once the virus is eradicated, to limit and control the spread of the virus upon re-introduction by regional vaccination and direct follow up by the use of DIVA serological assays.

a) Economic impact

PRRSV has a high prevalence in pigs worldwide. Pig losses due to PRRSV are extremely variable, both in extent and duration. It is important to discriminate between the effects of the disease in the epidemic and endemic phases (Done et al., 1996, Br Vet J). During epidemics with PRRSV strains in Europe, losses are due to (i) a decrease in the number of piglets reared per sow/year, (ii) an increase in farrowing interval and (iii) higher sow replacement rates and cause a reduction of 55% of the yearly income of a farm (Brouwer et al., 1994, Vet Q). Enzootic situations of PRRSV when the virus persists and circulates in the nursery and in the fattening units, are estimated to cause a loss of about 3€ per slaughter pig in Europe (Schuon et al., 2008, Proc 20th International Pig Veterinary Science Congress). During an outbreak in The Netherlands, a mean loss of €126 per sow was observed (Nieuwenhuis et al., 2012, Vet Rec). It was estimated in the United States that losses due to PRRSV cost the pork industry USD 664 million per year (Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers, MCHC, Holtkamp et al, 2013, Journal of Swine Health and Production) due to (i) reproductive problems, (ii) nursery losses, (iii) losses in the fattening units (Neumann et al., 2005). High abortion rates, mortality of pre-weaned piglets and respiratory disease in fattening pigs are the main features of this syndrome.

Recently, an outbreak of PRRSV occurred in November 2012 in Switzerland, traditionally PRRSV-free. It was detected after a German boar stud informed a semen importer about the detection of PRRSV during routine monitoring. The events demonstrate that import of semen from non-PRRSV-free countries even from negative studs poses a risk, because monitoring protocols in boar studs are often insufficient to timely detect an infection, and infections of sows/herds occur even with low numbers of semen doses.
The outbreak was eradicated successfully mainly due to the high disease awareness of the importer and because immediate actions were taken before clinical or laboratory diagnosis of a single case in the country was made. To minimize the risk of an introduction of PRRSV in the future, stricter import guidelines for boar semen have been implemented. Despite the early detection and control of the outbreak, its economic implications were still substantial. A total of 89 farms had to be put under animal movement restrictions and investigated, and on one farm, all animals (240 breeding animals and approximately 1700 piglets) had to be culled (Nathues et al., 2014, Transbound Emerg Dis).

Emergence of highly virulent PRRSV strains has caused a real economic disaster in Asia. Mortalities of up to 30-50 % were not an exception. PRRSV is also negatively affecting economics by trade restrictions in between countries. The adaptable, efficacious, inactivated vaccines developed in PoRRSCon will contribute to reduce the PRRSV-related losses.

b) Social impact

Highly virulent PRRSV is causing a devastating effect on pig production and the food supply in several low-income countries in Asia. Solving the PRRS problems in Europe and Asia in a joined Europe/Asian project, does not only help to solve economic losses due to PRRSV in Europe and Asia, but also to assure the availability of pig meat particularly in Asia. Controlling PRRSV in Asia has thus a strong social impact. Further, the interactions of European and Asian partners has given an open European-Asian platform of scientists working on viral diseases and vaccine development. This stimulates common research.

c) Scientific Impact

Partners of PoRRSCon are performing frontline research on PRRSV. The interdisciplinary approach of top research groups from universities, institutes and industry performing work on molecular epidemiology, virology, immunology, vaccine development and serology guarantee new discoveries ranging from new insights in the characteristics of PRRSV strains, molecular and cellular pathogenesis of PRRSV infections, immunobiology, vaccine development and strategies to control PRRS and even to eradicate PRRSV.

- A public virus bank that included European (Eastern and Western), Asian (from Vietnam and China) and American isolates encompassing both genotypes was created by our Polish partner, an OIE reference laboratory for PRRSV. Centralization of the data is crucial for allowing easy access by the whole scientific community.

- New PRRSV strains from both genotypes and from different subtypes were sequenced and characterized, and used to test detection by available commercial kits. While most of the serological methods characterized PRRSV strains with satisfactory performance, significant differences regarding qualitative diagnostics as well as analytical sensitivity were observed between the real-time RT-PCR assays. These findings emphasize that continuous monitoring of PRRSV genetic diversity, evaluation of diagnostic tests and updating primers and probes sequences are necessary to allow proper diagnostics. Close cooperation between diagnostic laboratories and the manufacturers of diagnostic PCR kits would be beneficial for both sides. A limited degree of cross protection/cross reaction between subtype 1 and subtypes 2-4 isolates make it necessary to test the cross antigenicity/neutralising capacity of any new vaccine entities prior to marketing. Because available commercial vaccines had not been tested yet against the virulent strains from Eastern Europe, we evaluated the efficacy of a commercial attenuated European subtype 1 PRRSV vaccine upon challenge with an East European subtype 3 PRRSV strain and showed that vaccination of pigs with an attenuated European subtype 1 vaccine provides only a partial protection against a subsequent exposure to the highly pathogenic East European subtype 3 PRRSV strain Lena (Trus I et al., 2014, Vaccine).

- The comparative pathogenesis of low and highly virulent PRRSV strains has provided information on the basis of the differences in targets of replication and effects induced between the low and highly virulent PRRSV strains.

- The virulence of PRRSV was analysed. Two virulence genes of highly virulent Chinese American type PRRSV strains were identified. This information will help in attenuating PRRSV strains and the development of vaccines by reverse genetics.
• Screening of the endometrium/fetal placenta for virus replication and virus-induced changes proved to be a useful tool to study the pathogenesis of congenital PRRSV infection and validate the PRRSV vaccine performance. Hence, an advanced protocol was proposed to test the PRRSV vaccine efficacy. Also, requirements were summarized for primary testing of PRRSV vaccines in pregnant animals which may be helpful in development of a monograph (Karniychuk et al., 2012, Theriogenology).
• The efficacy of innovative, autogenous inactivated PRRSV vaccines was compared with the one of commercial vaccines against homologous and heterologous challenges (Geldhof et al., 2012, BMC Vet Res). Inactivated farm-specific PRRSV vaccines can be useful tools to boost PRRSV-specific humoral immunity in sows and reduce viremia in weaned piglets. Also, significant progress was achieved in the development of attenuated marker HP-PRRSV vaccines that fully protect piglets from lethal challenge by highly pathogenic PRRSV while allowing DIVA (Wang et al., 2013, Virus Res).
• Technical achievements of the project that will assist scientists worldwide, include: (1) the development of fast and robust methods for long range RT-PCR amplification (Kvisgaard et al. 2013, Virol. Methods), (2) a technique using next generation sequencing to sequence PRRSV directly from primary material/cell culture without any prior sequence knowledge (Lu et al. 2014, Virol J), (3) the knowledge that monocyte-derived macrophages can be used as a PRRSV infection model instead of alveolar macrophages, reducing the number of pigs required for the collection of cells (Garcia-Nicolás et al., 2014, Virus Res), (4) an advanced protocol proposed to test the efficacy of PRRSV vaccines (Karniychuk et al., 2012, Theriogenology), (5) a published general review on virus inactivation (Delrue et al., 2012, Expert Rev Vaccines), and (6) the release of new PRRSV sequence data.

2. Main dissemination activities

Dissemination of scientific results

A total of 75 peer-reviewed scientific publications acknowledging the PoRRSCon funding have been generated by the Consortium and some additional ones are in preparation. The full list of publications is indicated below and is available also on the project website, sorted by relevant work packages. This website was set up and updated after every consortium meeting by the project manager. More than 160 other dissemination activities have taken place, under the form of posters (51), oral presentations to a scientific event (91), oral presentations to a wider audience (12), organisation of international congresses (2), presentations, exhibition, PhD thesis and other publications. The coordinator took actions to represent PoRRSCon at different veterinary and pig producer congresses like the International Pig Veterinary Society (IPVS) and gave the latest updates on the scientific output.

Two international scientific meetings were organized by PoRRSCon partners: one in 2013 (International Porcine Reproductive and Respiratory Syndrome (PRRS) symposium, May 20-22, 2013, Beijing, China; hosted by Prof. Hanchun Yang, CAU, China) and one in 2014 (XIIIth International Nidovirus Symposium, June 1-6, 2014, Salamanca, Spain; hosted by Prof. Luis Enjuanes, CSIC, Spain). The next International Porcine Reproductive and Respiratory Syndrome (PRRS) congress will be hosted by Prof. Hans Nauwynck, UGent and organised in Ghent, Belgium on June 3-5, 2015.

Cooperation with other projects/programs and Training impact

During the project, exchange of students has activated the dissemination of techniques and knowledge in between the partners of PoRRSCon in the field of virology (molecular and cell biological), immunology (cytokine research, immune responses, vaccinology), animal experiments (ethical issues, manipulations, tissue collection) and genomics. Most of the PoRRSCon partners are participating in other EU-funded projects and/or actions, which allows excellent interactions between the projects, for example, using short time scientific missions from the COST FA092 action (EuroPPRS.net, aiming at understanding and combating PRRS in Europe).

A PhD student from AHVLA participated in the pig experiment (WP4) at DLO-CVI to allow a better reproducibility of the same experiments that was repeated at AHVLA (with other viral strains, but same experimental protocol).
Another PhD student from AHVLA went to FDEA-IVI to participate to common immunological assays (WP5).

A PhD student from CReSA has been working for a few months at AHVLA.

As another example, the organization of a PRRSV RT-PCR ring trial was made in cooperation with EPIZONE Network of Excellence. Two subtype 2 isolates from Belarus, used for the first time in inoculation study linked to the NADIR project (DTU-VET and NVRI, project n° FP7-228394, "Porcine reproductive and respiratory syndrome virus (PRRSV): Virulence of East-European atypical isolates") provided unique tissue/monovalent sera collection. A reference set of samples representing all known PRRSV subtypes was assembled and used for proficiency tests and evaluation of diagnostic methods (see Tasks 2.2 and 2.3).

List of Publications of the PoRRSCon Consortium:

**Scientific Publications:**


54) Fabisiak M, Podgórsk a K, Skrzypiec E, Szczotka A, Stadejek T., 2013, Detection of porcine
cercovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in meat juice samples from Polish wild boar (Sus scrofa L.). Acta Vet Hung. Jul 16:1-8.


Other Publications:


3. Exploitation of the results

The observation that homologous BEI-inactivated vaccines can provide a predictable degree of protection against a specific virus variant suggests that such vaccines may prove useful in case virus variants emerge that escape the immunity induced by the attenuated vaccines. A farm-specific vaccine can induce virus neutralizing antibodies and offer partial protection upon homologous challenge and therefore may be used in addition to the commercially available vaccines on farms with PRRSV-related problems. The coordinator, together with PROVAXS, the business development center of a network of laboratories of
the University of Ghent, took care of the protection and exploitation of the results. Valorization of this new type of European (subtype 1) PRRSV inactivated vaccine that is safe, efficacious, and adaptable, that offers partial protection against viremia and reproductive failure, as well as protective colostral immunity, has been achieved.