



Infection of Piglets with the Porcine Respiratory and Reproductive Syndrome Virus (PRRSV): A Morphological Study

Rajcani J^{1*}, Banati F¹, Szenthe K², Gyurjan I³, Stipkovits L² and Szathmary S³

¹RT-Europe Non-profit Research Centre, Var ter 2/E, Mosonmagyaróvár, H-9200, Hungary

²Carlsbad Research Organization, Szabadság utca 2, H-9244 Ujronafo, Hungary

³Galenbio Ltd, Lucsony utca 15-17, Mosonmagyaróvár, H-9200, Hungary

*Corresponding author: Rajcani J, RT-Europe Non-profit Research Centre, Var ter 2/E, Mosonmagyaróvár, H-9200, Hungary; E-mail: Julius.Rajcani@savba.sk

Abstract

Together 28 piglets (aged over 2 months) were infected with 10^5 TCID₅₀ of porcine respiratory and reproductive syndrome virus (PRRSV) into both nasal nostrils using an inoculum of 2x150 microliters (together 300 μ l). Another 9 piglets served as uninfected controls. On days 11 and 18 post-infection (p.i.), either 12 and/or 16 infected animals were sacrificed to take the tissue samples from tonsillar area, each lung lobe as well as the spleen and liver. At both intervals, blood was removed to isolate white blood cells and to obtain serum for specific antibody detecting ELISA assay. At histological examination, the typical picture of usual interstitial pneumonia (UIP) was seen in the lungs of 23 out of 28 infected animals (82 %). In each such case, in the lungs the thickened inter-alveolar septa revealed widespread mononuclear (mainly lymphocyte) infiltration occasionally reaching an extensive intensity. By immunohistochemically staining for N-protein this was found in the ciliary epithelium cells lining the bronchial tree by nearly all piglets who developed UIP (in 21 out of 23 animals, i.e. in 91 %). In contrast, the squamous epithelium at the pharyngeal and/or tonsillar areas of same piglets was less frequently positive (in 13 out of 23, 57 %).

Keywords: Porcine respiratory and reproductive system virus (PRRSV); Usual interstitial pneumonia (UIP); PRRSV antigen detection

Introduction

The porcine respiratory and reproduction syndrome virus (PRRSV) has been classified into family *Arteriviridae* (order Nidovirales), along with the equine arteriitis virus and/or the lactate dehydrogenase elevating virus of mice [1-3]. The virions form small, enveloped particles (50-65 nm in diameter) harboring a relatively long (approximately 15 kb in size) single strand RNA genome [4]. The viral RNA (vRNA) is a positive-sense molecule with terminal cap at 5'-end and a poly-A repeat at 3'-end [5-7]. In the course of virus replication, the vRNA is copied as whole, being synthesized via a full length negative-strand intermediate. In the course of vRNA replication, a complete (genomic) minus strand is generated, which serves as template for the synthesis of the new vRNA. Also for viral mRNA synthesis, first a negative

sense RNA sequence is formed, from which the set of positive sense nested sub genomic (sg) RNAs is being transcribed. Thus, minus sense subgenomic (sg) RNAs are used as template for the synthesis of the functional positive sense sg mRNAs [23/26]. The vRNA has of 2 long open reading frames (ORF1a and ORF1b), which comprise about 75% of the total genome sequence. This portion of the genome specifies 14 non-structural proteins (nsp) formed by cleavage of the 2 corresponding translated polyproteins. Of special importance are the non-structural proteins 9 (nsp9) and 12 (nsp12), representing the vRNA replicase, termed also RNA-dependent RNA polymerase (RdRp). The rest of the genome encodes 7 structural proteins, out of which 5 are glycoproteins (designated GP2a/Gp2, GP2b/E, GP3, GP4 and GP5) in addition to the M (membrane) protein and the N nucleoprotein [4]. Both strands are complementary to each other

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and at their conterminal 3'-end and are equipped with a common leader sequence at their 5'-ends [27/28]. The viral genome is equipped with several (but at least two) conserved transcription regulatory sequences (TRS), located in the front of ORF1a and ORF2a which encode the structural protein GP2a and the envelope glycoprotein (Gp2b/E). Two different strains were isolated in the US (VR2332) and in Europe (Lelystad) revealing serologic as well as genomic differences [8-13]. Experimental infection with the PRRSV isolates can be lethal in newborn and/or 3-week-old piglets. A key event is the involvement of porcine alveolar macrophages, which represent an important target for infection allowing virus spread [14,15]. To date, at least two macrophage surface molecules are known as entry mediators: the sialoadhesin and CD163, a scavenger receptor [10]. The PRRSV-induced pneumonia is characterized by thickening of inter-alveolar septa due to infiltration with macrophages and by the presence of occasional inflammation and cell debris within alveolar cavity. The pneumocytes of type II which are lining the alveolar cavity may also be PRRSV positive along with the hyperplasia of per bronchial lymphatic tissue [24/25]. 3 The severity of lung lesions may vary from relatively mild to more extensive. The viral genotypes can differ in their pathogenicity, namely the Type 2 North American PRRSV induces more severe respiratory disease than type 1 European virus [16]. In this paper we describe the correlation of lung lesions as seen by standard histological examination in comparison with immunohistochemically detection of viral N-protein along with the results of serological tests for N-protein antibodies.

Materials and Methods

Virus. The North-American strain of PRRSV was cultured using the MARC-145 cell line (African green monkey kidney cell line derivative of MA-104, ATCC-CRL-12231) in EMEM medium at 37°C in the presence of 5% CO₂; the virus end point (TCID₅₀ titer) was tested in 96-well plates as described. (2014)31/32 and Ramakrishnan (2016)21/22, respectively. **Animals.** Pigs (together 28 infected animals) were inoculated into both nostrils with 105 TCID₅₀ of above mentioned PRRSV strain being administered in a volume of 300µl tissue culture supernatant. The nine (9) negative control animals were inoculated in a similar way but with a virus free culture medium; they were kept under carefully checked conditions of strong isolation to avoid any possible contact with the virus-inoculated piglets. Specimen sampling and histological examination. Blood samples were taken on days 0,2,4,6,8,10,15,17 post-infection for serological examinations. At given intervals (on days 11 and 18) post-infection (p.i.), the animals were succumbed and selected tissue samples (coming from each lung lobe, from both tonsils including adjacent pharyngeal area, from spleen and liver) were immediately

immersed into cold 10% neutral formalin, fixed for 24 hr and embedded into paraffin. Sections were cut in an amount to stick to at least 3 parallel slides. Those destined for the standard histological examination were stained by hematoxylin and erythrosine (HE), while the rest was handled by immunohistochemically staining. Briefly, the slides were rinsed in phosphate buffer saline (PBS) and then dehydrated in a series of corresponding reagents as described. The mix of commercial monospecific mouse ascites derived antibody against the N-protein of PRRSV had been purchased from 4rtilab and contained both, the SDOW-17 as well as the SR30-A reagents. Both anti-N antibody solutions were freshly mixed before use in an equal (1:1) ratio. In the second layer, an alkaline phosphatase labelled anti-mouse IgG antibody was applied. An additional parallel slide which had been included, was treated with the second antibody only (it served as staining control for detection of any putative non-specific background staining). 4 Saliva (oral fluid) collection. The oral fluid was pooled from each animal separately using the Civets suis oral fluid rope (IDEXX) ELISA titer measurements. The obtained saliva samples were examined for the presence of class IgA antibody specific against PRRSV using the Oral Fluids (IDEXX PRRS OF) kit as recommended by the manufacturer. In case of blood samples the positive control monospecific serum to class IgG antibody against the PRRSV was tested using the INgezim PRRS 2.0 ELISA kit (purchased from Eurofins) strictly following the recommendations of the manufacturer [17].

Results

As documented in the interalveolar septi in normal lung tissue are very thin in order to ensure the diffusion of oxygen into blood capillaries, where the erythrocytes circulate. Occasionally, a few mononuclear cells (mainly lymphocytes) might be seen in the peribronchial connective tissue. Surprisingly, in 4 out of 9 non-infected (control) piglets, a slight focal thickening of interalveolar septi was noted along with accumulation of a mild interstitial infiltrate consisting of mononuclear cells, mainly lymphocytes (Figure 1).

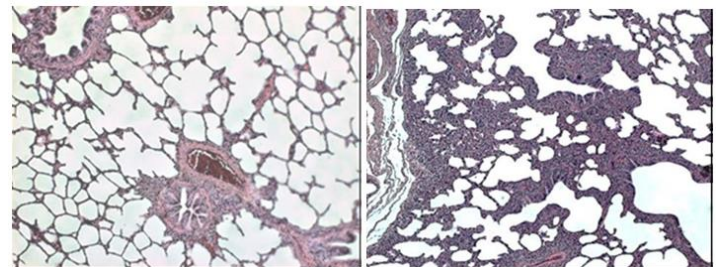


Figure 1: Histological picture of lung tissue in uninfected (control) piglets.

- In the left (piglet no. 5). The normal lung structure at low power view shows the thin interalveolar septa devoid of infiltrate; the peribronchial (and/or perivascular) connective tissue reveals a minimal infiltrate of very few mononuclear lymphocytes (magn x100).
- In the right (piglet no. 2). Unlike to Fig. 1A, this area lung tissue area shows thickened interalveolar septa due mild accumulation of mononuclear lymphocytes. Similar mild (M) non-specific (NS) interstitial infiltrate (II) was found in the lungs of 5 out of 9 uninfected controls.

As expected, staining for PRRSV in any control lung tissue sample was negative including areas revealing the above mentioned mild interstitial infiltrate (Figure 1b).

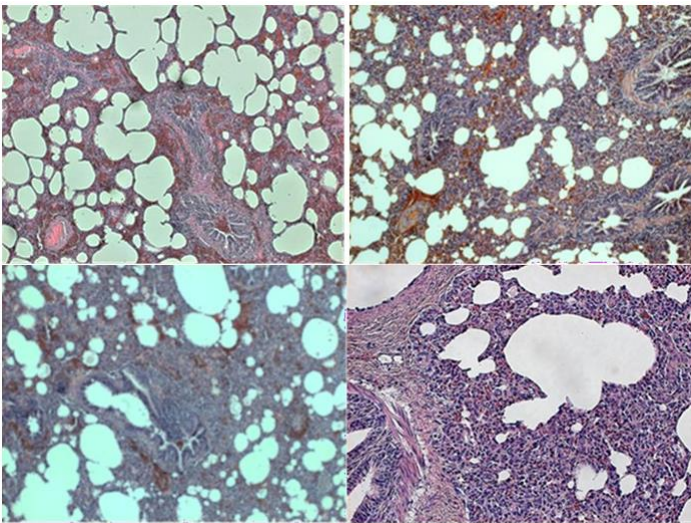


Figure 2: Histological findings in the lungs of PRRSV infected piglets.

- In the left above (piglet no. 16). An area of the lung tissue in the infected animal showing mild focal thickening of the interalveolar septa and dilatation of small vessels (magn x100).
- In the right above (piglet no. 16). In contrast to the area shown in the left, this one reveals typical UIP with severe thickening of interalveolar septa and an abundant mononuclear interstitial infiltrate along with dilatation and hyperemia of the small blood vessels.
- In the left below (piglet no. 25). The lung tissue of an animal who developed typical UIP showing a widespread mononuclear infiltration of the interalveolar septa and peribronchial connective tissue (magn. x100).
- In the right below, the same piglet (no. 25). The mononuclear infiltrate in the peribronchial area consists mainly of lymphocytes; the thickening of interalveolar septa is prominent (magn. x 240).

As expected, in the majority of infected animals (23 out of 28, 82%) the lung tissue revealed the typical picture of usual

interstitial pneumonia (UIP). In these, the interalveolar septi were thickened along with the presence of a rich mononuclear cell (lymphocyte) infiltrate. In addition, in UIP the capillaries of the alveolar wall were widened along with occasional focal bleeding into the alveoli in result to an injury of endothelial cells. High power view of altered areas confirmed that the interstitial infiltrate consisted mainly of lymphocytes. Occasionally (in example by piglet no. 40) the infiltrate in question was so extensive that the original lung structure became altered or completely different so that it could not be recognized either (Figure 3).

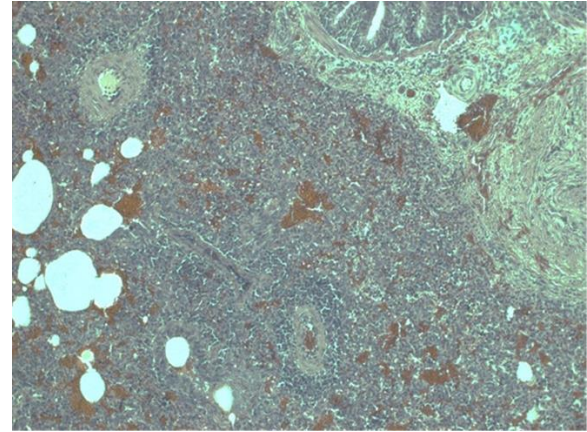


Figure 3: Severe interstitial pneumonia due to PRRSV infection.

Staining with anti-N antibody showed the presence of PRRSV antigen predominantly in the columnar ciliary epithelium lining the bronchial tree. Details demonstrated the presence of viral antigen in the cytoplasm of acinus cells building the small mucous glands, situated below the lamina muscularis of the bronchial wall. The alveolar lining was rarely positive for the N-protein. Occasionally, the type II alveoli lining cells could be found harboring the N-protein (Figure 4).

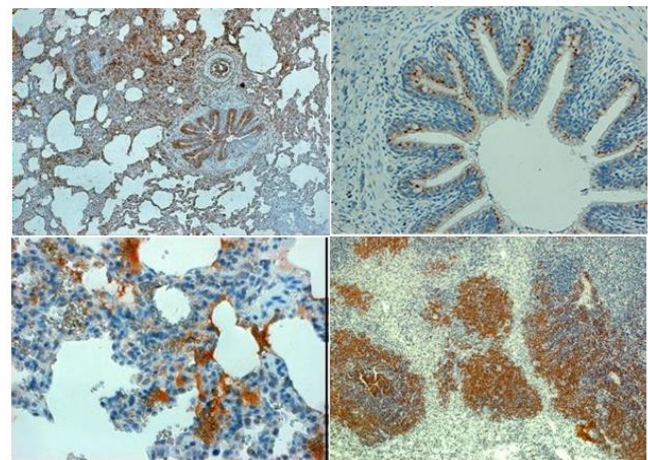


Figure 4: Staining for N-antigen in the respiratory tract and spleen of PRRV infected piglets.

- In the left above (piglet no. 34). The lung tissue shows overwhelming staining for N-protein, namely the bronchial epithelium, the parabronchial mucous glands and the flat lining of the aveolar wall (magn. 80x).
- In the right above (piglet no. 12). An intrapulmonar bronchus enlarged: the N-protein can be seen in the cytoplasm of ciliary epithelium cells lining the bronchial tree; a few negative goblet cells can be seen as well (magn. 120x).
- In the left below (piglet no. 45). The N-protein is present in the cytoplasm of cells lining the alveolar wall and in several mononuclear phagocytes invading the interalveolar septi (magn. 400x).
- In the right below (piglet no. 44): the white pulp of the spleen shows lymphatic follicles formed mainly by lymphocytes which are positive for N-protein (magn. x120) (Figure 5).

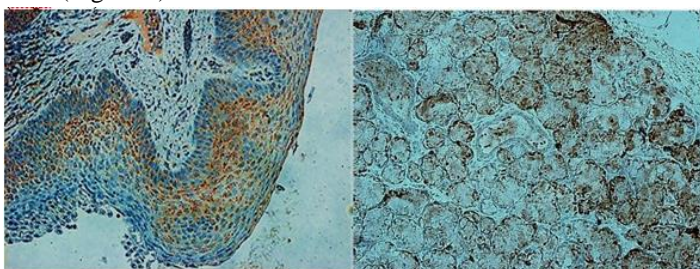


Figure 5: N-protein in pharyngeal epithelium and salivary gland of PRRSV infected piglets.

- In the left (piglet no. 26). The N-protein in tonsillar area can be found mainly within the cytoplasm of proliferating squamous epithelium cells at both suprabasal and/or intermedial layers; a few basal epithelium cells are positive as well (magn. 220x)
- In the right (pig no. 13). The N-protein can be seen in the acini of submandibular salivary gland (magn. 220x).

The N-protein could be also found in the cytoplasm of alveolar macrophages moving from intraalveolar space across the interalveolar septa to the local lymphatic capillaries in order to reach the sinuses of regional lymph nodes. Nevertheless, in some piglets the local peribronchial lymph nodes did not hamper the virus spread, so that via the infected virus carrier cells viremia has developed (see below) involving remote organs such as spleen and/or liver. In the spleen, the reticular cells of regional sinuses could be found positive along with the lymphocytes forming the lymphatic follicles. In the liver, the N-protein could be detected in the cuboid epithelium cells forming the intrahepatal biliary ducts. Outside of lung tissue, the N-protein of PRRSV was found especially in the nonhornified squamous epithelium of the pharyngeal area including that over tonsils. Here the virus antigen occupied the deeper layers of stratified epithelium, namely the multiplying parabasal cells as well as those in the medium layer.

The virus was also found in salivary glands, namely in the submandibular gland, where the N-antigen was harbored in the cytoplasm of acinus cells. Noteworthy, the presence of viral N-antigen in the acinar cells of salivary glands was relatively rare in comparison to the tonsillar and/or pharyngeal squamous epithelium, which still were relatively more frequently positive. However, the real incidence of N-protein in the former was difficult to assess, since the sections of salivary glands could not be regularly found. By testing the occurrence of the virus in the blood, viremia was found to peak on day 6. While by day 10 p.i. already no virus could be detected. As mentioned in the section Materials and Methods, development of the specific class IgG anti-N antibody response was followed by ELISA. As documented on (Figure 6B), unlike to virus presence, no N-specific serum antibodies occurred before day 11 p.i., but on day 18 p.i. they were detected in high levels. The low amount of serum antibodies on day 11 might be related due to their binding to virus particles. Contemporarily, first traces of the anti-N protein class IgA antibodies in the saliva also appeared from day 11. As the levels of blood lymphocytes concerns, these started to decrease from day 4 post-infection, returning back to the original level on day 10. The kinetics of the polymorphonuclear leukocytes (i.e. blood neutrophils) in the blood revealed a similar outcome (data not shown) [18-20].

Discussion

As a member of the family Arteriviridae, the porcine respiratory and reproductive syndrome virus (PRRSV) belongs to the order Nidovirales together with the Coronaviridae and Roniviridae families, [26/27]. PRRSV was originally divided into European type 1 and North American type 2 genotypes. Later on, the East European PRRSV isolates have been found to be of the European genotype, but forming different subtypes [6]. A novel virus, namely the 6 Belarusian strain Lena, has been recently characterized as a highly pathogenic East European subtype 3, which differs from European subtype 1 Lelystad and North American US5 strains at genetic as well as antigenic levels [11]. A novel PRRSV strain isolated on a French pig farm has been recently identified to represent a modified recombinant consisting from two genotype 1 live vaccine strains, namely the VP-045BIS and DV ones [22/23]. For example, the kinetics of antibody responses directed against nonstructural virus coded proteins (nsp) can be analysed in pigs experimentally exposed to the virus [23/24]. In such case, high antibody reactivities especially against nsp1, nsp2, and nsp7 were noted. Among the latter, nsp7 recombinant protein based ELISA showed good sensitivity and specificity most suitable for diagnostic development especially for identification and differentiation of type 1 and type 2 PRRSV. Several nonstructural proteins (such as nsp1, nsp2, nsp5, nsp7 nsp9, nsp10 and nsp11) have been implicated in the induction of

IFN- γ and also in the development of the cell-mediated immune response [17]. On other hand, the induction of neutralizing antibodies (NAs) may be delayed and/or their levels may remain low, which is not only the problem of early diagnostic, but is also of importance regarding effective virus elimination. NAs may

protect against disease if present in sufficient quantities before infection, but they do not seem to be essential for clearing virus in blood during the course of the infection. PRRSV is able to modulate innate responses, probably through the regulation of IFN- α and IL-10 responses [14].

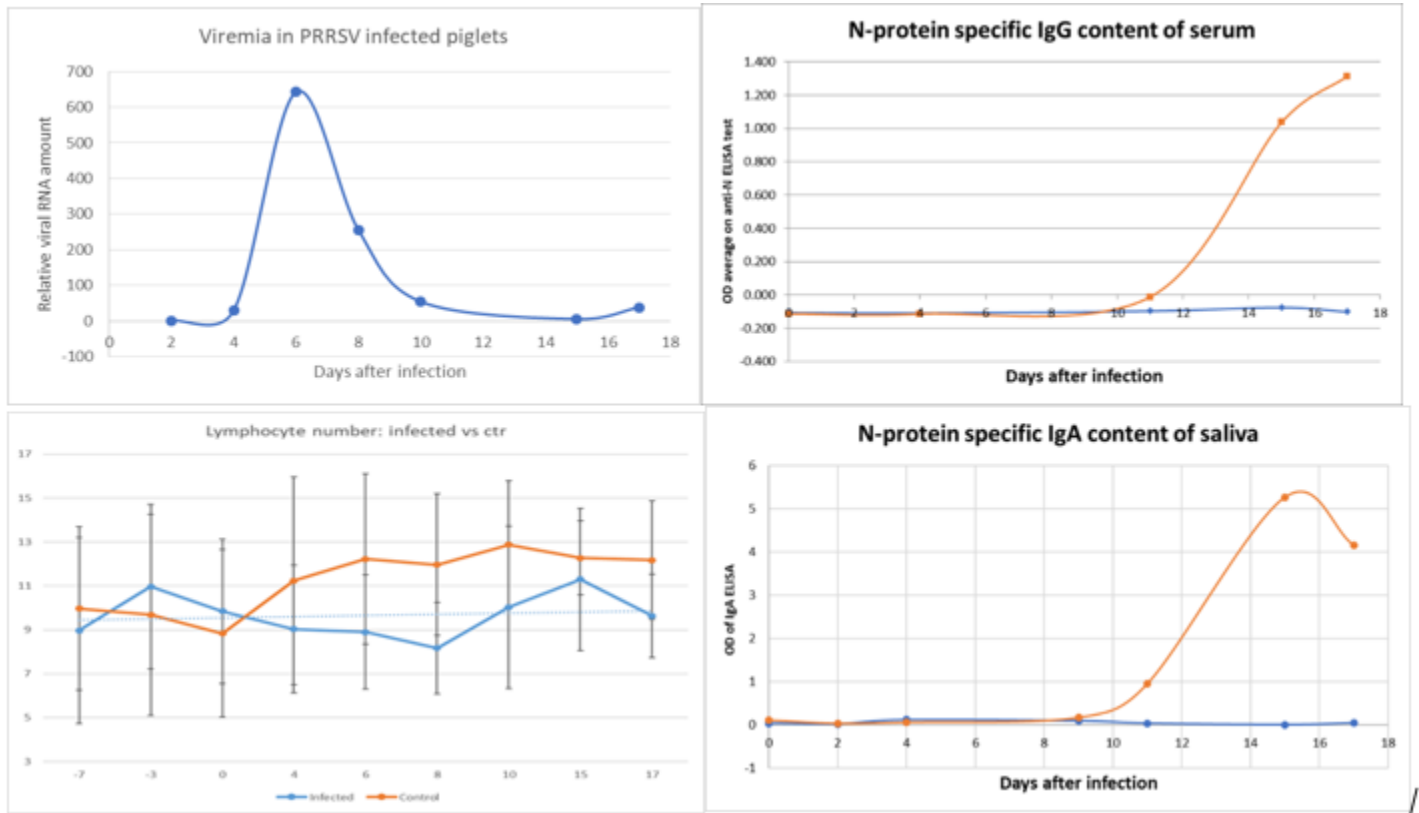


Figure 6: Detection of vRNA in the serum of infected pigs in comparison with the specific antibody response. Viremia has been detected on days 6 and 8 post-infection, while the anti-N protein class IgG antibody began to rise from day 11 reaching high levels on day 15 and/or 18 post-infection. The local class IgA antibody formation in the saliva showed a kinetics similar to that found in serum. The blood lymphocyte number started to decrease from day 4 and remained low until day 10, later on returning to the early post-infection level on day 14. The blood neutrophils revealed a similar kinetics (data not shown).

Table 1: Histological lesions and the presence of N-antigen in infected piglets.

Animal	PRRSV	Results
Uninfected	None	No N-antigen was seen neither in bronchial epithelium (0/9) nor in tonsils (0/9).
Infected	Yes	Histological picture of UIP* has developed in the lungs of 23 out of 28 infected piglets; the N-antigen was seen in the bronchial epithelium of 21 infected animals, while their alveolar lining was positive in 5 cases only. Outside of lungs, the N-protein was detected in the tonsillar epithelium of 13 out of 28 infected piglets; in contrast, the spleen was positive in 3 piglets and the salivary gland in 1 animal only.

The PRR syndrome caused by this virus in piglets is characterized with high mortality, reproductive failure (late-term abortions and stillbirths, premature farrowing, mummified pigs in pregnant sows) and a severe respiratory disease (interstitial pneumonia). The disease occurring in the nursery and among growing/finishing piglets causes significant economic losses to the swine industry worldwide [19/20]. The corresponding virus replicates mainly in the porcine alveolar macrophages (PAMs) and dendritic cells (DCs) [20/21] [21-23]. The virus also causes persistent infection eliciting antibody dependent enhancement (ADE) and occasional immunosuppression. Numerous results suggest that PRRSV may utilize multiple strategies of replication and spread in the infected pigs, including subversion of the host innate immune response, inducing an anti-apoptotic and anti-inflammatory state as well as developing ADE. The genome wide host transcriptional responses were studied using the North American strain CH1a; the results documented multiple replication strategies including subversion of the host innate immune response as well as inducing an apoptotic state [25/26]. As described above, PRRSV predominantly replicates in the lung alveolar macrophages and can cause prolonged viremia as well as persistent infections lasting for months. This virus strongly modulates the host's immune response including changes in gene 7 expression, especially inhibiting type I interferons (IFN- β). Regarding to cell-mediated responses, the development of PRRSV-specific gamma interferon-secreting cells (IFN γ -SC) and interleukin 4-secreting cells (IL4-SC) was examined by ELISPOT assay using extracts of peripheral blood mononuclear cells (PBMC). Using this technic, no IFN γ -SC was detected until day 14 p.i., whereas for IL4-SC no such differences were seen. Concurrently with the onset of viremia and the development of clinical signs, the serum haptoglobin levels and interleukin 10 (IL-10) increased significantly in the PRRSV stimulated PBMC-culture supernatants. These results are compatible with the model of pathogenesis in which the immune response does not fully control the outcome of infection [8]. The PRRSV induced immunosuppression might mediate apoptosis of infected cells, which causes depletion of immune cells and induces an anti-inflammatory cytokine response due to which the host is unable to eradicate the primary infection. The initial antibodies do not confer protection and can even be harmful by mediating an antibody-dependent enhancement (ADE), since they can facilitate the virus entry of into targets cells in vitro. To characterize the humoral immune response direct enzyme-linked immunosorbent assays (ELISA) can be used including different mainly recombinant PRRSV antigens. For example, the kinetics of antibody responses directed against nonstructural virus coded proteins (nsp) has been analysed in pigs experimentally exposed to the virus [5]. In these experiments, high antibody reactivities

against nsp1, nsp2, and nsp7 were noted. Among the latter, the nsp7 recombinant protein based ELISA showed good sensitivity and a degree of specificity most suitable for diagnostic development, namely for identification and differentiation of type 1 from type 2 PRRSV. In contrast, a minitope in the N-terminal ectodomain of structural glycoprotein GP312, was found to contain the immunogenic SRHDHIIH motif, which are conserved consensus sequences at amino acid positions 156 to 161 [24,25]. Non-structural proteins (such as nsp5, nsp9, nsp10 and nsp11) additional to above listed ones, have been also implicated in the induction of IFN- γ and also in the development of the cell-mediated immune response. On other hand, the induction of neutralizing antibodies (NAs) may be delayed and/or their levels may remain low, which is not only the problem of early diagnostic, but is also of importance regarding effective virus elimination. NAs may protect against disease if present in sufficient quantities before infection, but they do not seem to be essential for clearing virus in blood during the course of the infection. PRRSV is able to modulate innate responses, probably through the regulation of IFN- α and IL-10 responses [17]. The PRRSV antigen(s) may occur in blood associated with exosomes as documented in viremic as well as non-viremic piglets [18]. However, the virus predominantly replicates in the 8 lung alveolar macrophages and can cause prolonged viremia as well as persistent infections lasting for months. This virus strongly modulates the host's immune response including changes in gene expression, especially inhibiting type I interferons (IFN- β). Regarding to cell-mediated responses, the development of PRRSV-specific gamma interferon-secreting cells (IFN γ -SC) and interleukin 4-secreting cells (IL4-SC) was examined by ELISPOT assay using extracts of peripheral blood mononuclear cells (PBMC). Using this technic, no IFN γ -SC was detected until day 14 p.i., whereas for IL4-SC such difference was not seen¹². Concurrently with the onset of viremia and the development of clinical signs, the serum haptoglobin levels and interleukin 10 (IL-10) increased significantly in the PRRSV stimulated PBMC-culture supernatants. These results are compatible with the model of pathogenesis in which the immune response does not fully control the outcome of infection⁸. As already mentioned, the replication and spread of PRRSV in the body subverts the host innate immune response. In addition, when high jacking the host lipid metabolism and inducing an anti-apoptotic as well as an anti-inflammatory state, it may suppress the expression of some enzymes and their regulators, such as the serine proteinase inhibitor 2 (SPI 2) and IFN- α . Furthermore, it down-regulates the expression of pro-apoptotic genes, such as the B-cell lymphoma 2 (Bcl-2) antagonist/killer (BAK) and/or the Bcl-2 associated X (BAX). The product of the latter gene can be seen predominantly in the cytosol, but more precisely, it is localized within the outer

mitochondrial membrane. Therefore, toxic mitochondrial pores can occur in response to cellular stress. Furthermore, the APR-1, i.e. the Adenomatous polyposis coli (APC) protein, which is a component of the Wnt signaling system, may be down-regulated along with the microtubule-associated protein SARP3 (several ankyrin repeat protein 3). Both of them were shown to interact with all isoforms of PP1 (protein phosphatase 1). Infection with PRRSV result in fever and tissue inflammatory response, as indicated by high expression of proinflammatory cytokines and chemokines, including adhesion molecules, inflammatory enzymes as well as their receptors, such as IL-1 β , IL8, SELL, ICAM, CCL2, CXCL9, CXCL10, B2M, proteasomes and cathepsins. This was compounded by cell death and elevated expression of NFKBIA, XAF1, GADD45A, perforin, granzymes, and cytochrome C, coupled with increased ROS-mediated oxidative stress, as indicated by up-regulated expression of cytochrome B245. Therefore, PRRSV infection may have result into an excessive immune response and an inflammatory reaction, both contributing to tissue damage¹² (Figure 6) (Table 1) [25-32].

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