

**Szent István University
Postgraduate School of Veterinary Science**

**COMPLEX EXAMINATION OF PORCINE CIRCO VIRUS TYPE-2 AND PORCINE
CYTOMEGALOVIRUS CAUSING DISEASES IN DOMESTIC PIG BREEDS**

Brief Summary of Doctoral Theses

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Introduction

Recently, several new viral infections of swine emerged, which have common characteristics in targeting the immune system and thus, causing serious economical losses through secondary bacterial infections. These viruses are the Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), the Porcine Circovirus type-2 (PCV-2) and the Porcine Cytomegalovirus (PCMV). All of these viruses alter (suppress or increase) the activity of cells playing pivotal roles in the animals' defence mechanisms. The consequent serious damage to multiple body organs can kill the animals by themselves, but the secondary bacterial infections are even more devastating. Further common characteristic feature of the above-mentioned infections is that their epidemiology, pathogenesis and the interaction with the organism is not yet, or only partly known. In this research work PCV-2 and PCMV viruses were studied in this aspect.

PCV-2 infection shows up in at least two different clinical and pathological forms. The first one is the so-called Postweaning Multisystemic Wasting Syndrome (PMWS), which occurs mainly in pigs aged 50-60 days. The disease causes wasting, immuno-suppression and consequential bacterial infections. Another PCV-2 related disease is the Porcine Dermatitis Nephropathy Syndrome (PDNS), which comes with haemorrhagic-necrotizing dermatitis, immunocomplex glomerulonephritis and interstitial nephritis. The virus generally multiplies in lymphocytes and macrophages, accordingly, it causes moderate to severe lymphocyte depletion, and interstitial inflammation in several organs, characterized by lympho-histiocytic infiltration and macrophage proliferation. Presence of PCV-2 infection can be detected using serological, molecular-biological or virological methods. The alterations caused by the virus in lymphocyte populations and macrophages can be conveniently detected by flow cytometry and in situ hybridization (ISH).

The PCMV infection is the cause of the so-called inclusion body rhinitis. The first Hungarian occurrence of the disease was described in 2002 by Deim et al. PCMV belongs to *Betaherpesvirinae*, it also multiplies in macrophages and lymphocytes. This viral infection causes clinical disease with high mortality mainly in suckling piglets. The disease is characterized by lympho-histiocytic inflammation of the respiratory tract and of lymphoid organs. The virus infection can be detected by serological, molecular biological and virological methods.

In my planned research first of all I intended to collect data on the distribution of the above-mentioned viruses in Hungarian swine herds, My aim was also to estimate the losses attributable to these infections. To accomplish these aims, I used statistically valid random sampling of Hungarian swine herds, and attempted to confirm the presence or absence of the viruses through the use of serology, necropsies, histopathological examinations and molecular biological methods. The second major part of my work was related to the influence of PCV-2 and/or PCMV virus on the porcine immune system. More specifically, I wanted to study the alterations caused by these viral infections in the peripheral blood mononuclear cells and pulmonary macrophages. The effects of separate and simultaneous PCV-2 and PCMV infections were studied using an experimental infection followed by histopathological, immunohistochemical, molecular biological studies. Similar analyses have not been done yet in Hungary and there are low numbers of publications related to this references even from abroad are few. I certainly hope, that my planned research work would result in new and important data on the effect of certain viral infections on the porcine immune system. Such data could help in diagnosing and preventing PCV-2 and/or PCMV infections.

Materials and methods

All samples went through the same diagnostic process: gross section, sample collection, formalin fixation and hematoxylin-eosin staining, bacteriological examination, and polymerase chain reaction (PCR) testing for the presence of PCV-2 and PCMV. The tissue samples were fixed in 10 % formalin, embedded in paraffin wax and sections (4 µm) were stained with haematoxylin and eosin.

***In situ* hybridization.** A sensitive, non-radioactive *in situ* hybridization test was used to visualize PCV-2 and PCMV DNA in selected tissue paraffin sections. A 567 bp long sequence (27360-27927nt AF318573) of ORF 20, of a protein conserved in gammaherpesviruses was amplified by PCR with digoxigenin-labelled UTP (DIG Probe Synthesis Kit Roche, Basel, Switzerland). The DIG-labelled BoHV-4 specific DNA molecules were used as probes in the *in situ* hybridization, and were detected using anti-DIG antibodies conjugated to horseradish peroxidase. The products were visualized with NBT/BCIP (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche, Basel, Switzerland). This method showed excellent sensitivity since the ISH signal was clear and the background staining was negligible.

An **immunohistochemical** method was used to detect PCV-2 in formalin-fixed and paraffin-embedded tissue samples from fetal and pig organs. Briefly, after dewaxing of sections, antigen retrieval was performed with 0.1% protease XIV solution (Sigma Aldrich Co.) at 37 °C for 10 min or in citrate buffer (pH 6.0), by heating in a microwave oven (750 W) for 20 min. The samples were incubated in 3% H₂O₂ solution for 10 min and then blocking was performed with a 2% solution of skimmed milk powder for 20 min. The sections were incubated with the primary antibodies at 4°C for a night with the dilution of 36A9 (Ingenasa, Madrid). The binding of antibodies was detected with a horseradish-peroxidase-labelled streptavidin-biotin kit according to the manufacturer's instructions (Universal LSAB2 Kit-HRP, Dako Co., Glostrup, Denmark). The sections were treated with 3-amino-9-ethylcarbazole solution (Sigma Aldrich Co.) also containing 0.01 % H₂O₂, at room temperature for 10 min, counterstained with Mayer's haematoxylin for 20 sec, covered with glycerol-gelatine. Tissue sections infected with the respective agent were used as positive controls.

All of the samples were tested for the presence of PCV-2 and PCMV by **PCR**. DNA was extracted from homogenates of spleen, liver, kidneys, mesenteric lymph nodes, lung, myocardium and thymus of 5 piglets from each litter with AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, California, USA). Specific primers were designed to amplify PCV-2 and PCMV target sequences using the published GenBank sequence data. ReadyMixTM PCR Reaction Mix (Sigma, St. Lois, MO, USA) was used for the PCR reactions. PCR amplified products were visualized by staining with 0.5 µl/ml ethidium bromide in a 1,5 % agarose gel.

Results

3356 pigs and 785 fetuses were sent into the Central Veterinary Institute for diagnostic investigation in the period of September 2003-September 2005. One thousand one hundred ninety samples were positive to PCV-2 out of 1250 pig organs.

PMWS was diagnosed in 789 (63.12 %) of the cases, whereas the PCV-2 presence was detected by PCR in all cases.

Characteristic lesions of acute and chronic stadium in the PMWS

	Acute	Chronic
spleen: lymphocyte depletion	+	+
lymph nodes: lymphocyte depletion necrosis, inclusion body	+ -	+ +
kidney: infl. interstitial	-/+	+
liver: infl. interstitial	-/+	+/-
lung: infl. interstitial syntitial giant cells edema	+ - +	+ + +/-
hydrothorax	+	-/+
brain: perivascularitis	-/+	-/+
wasting	-	+

PDNS was diagnosed in 34 (2.72%) cases from 1250 pigs.

Prevalence and occurrence of the histopathological lesions in the PDNS

Skin	Kidney	Liver	Lung	Brain	Lymphonode, spleen
21/21; 100%	21/21; 100%	9/21; 42.9%	15/21; 71.4%	3/21; 14.3%	15/21; 71.4%

In „typical” PDNS cases main histopathological changes were seen in the skin, kidneys, liver, lymph nodes, spleen and brain. In the skin samples multi-focal mononuclear vasculitis with thrombotization and neutrophil granulocytic infiltration were detected, in the kidneys acute exsudative glomerulonephritis, lympho-histiocytic interstitial nephritis, and in more advanced cases fibrosis with tubular dilation and atrophy were seen. Lympho-histiocytic interstitial pneumonia and hepatitis, focal mononuclear vasculitis in the brain and lymphocytic depletion in the lymphoid organs were also detected with varying frequencies. By *in situ* hybridization PCV-2 nucleic acid could be detected in the cytoplasm of lymphocytes and histiocytes (macrophages) of inflammatory infiltrates, in the endothel cells of dermal blood vessels, in the renal glomeruli, in the epithelial cells of visceral and parietal lamina of Bowman’s capsule and in the tubular epithelial cells.

The vertical transmission of PCV2 does occur. It neither replicates in the porcine fetuses nor causes abortion and stillborn piglets. Although gross lesions were not common in the fetal organs, there might be histopathological lesions associated with the viral infection. Lymphocyte depletion of lymph nodes and spleen, together with macrophage proliferation of lymphoid tissues and thymocyte depletion are characteristic lesions, which belong to the basic features of PMWS in affected piglets. These findings have tight relationship with the decrease

of B- and T-cells and consequently the immunodeficiency. After all, it is obvious that the thymus has serious and determinative role in the etiology of immune-system insufficiency and wasting syndrome.

I first diagnosed the porcine **inclusion body rhinitis** in Hungary. I recognized the inclusion body rhinitis in 15 pig herds in 2004-2005. Porcine cytomegalovirus DNA could be detected by specific PCR, by ISH, and by transmission electron microscopy in glandular epithelial cells of the nasal mucosa, in lacrimal glands and in pulmonary and hepatic lymphocytes and macrophages.

Possible predisposing factors of the presumed PCMV-infection were the large scale replacement of breeding sows, unfavorable conditions in the nursery (inadequate supplemental heating provided, large, draughty nursery rooms without proper insulation).

Since the first detection of PCMV in clinical cases of piglet mortality, I have encountered another similar problem on a different farm, not related to this one. Also, we have accidentally detected basophilic inclusion bodies in nasal glandular epithelial cells of suckling piglets from several herds, not reporting such large scale mortality. Based on these findings, PCMV infection is probably widespread in Hungarian swine herds, with occasional outbreaks of inclusion body rhinitis.

Main results

1. I collected sufficient data on the distribution of the aforementioned viruses in Hungarian swine herds.
2. I recognized the prevalence and occurrence of the PCV-2-caused diseases in domestic pig breeds.
3. I diagnosed the PMWS in wild boars.
4. I made comparative examinations between acute and chronic PMWS.
5. I developed in situ hybridization methods for PCV-2 and PCMV.
6. I developed immunohistochemical methods for PCV-2
7. I obtained new and important data on the effect of certain viral infections on the porcine immune system by ISH and IH.
8. PCV-2 nucleic acid was detected in the cytoplasm of lymphocytes and histiocytes (macrophages) of inflammatory infiltrates, in the endothel cells of dermal blood vessels, in the renal glomeruli, in the epithelial cells of visceral and parietal lamina of Bowman's capsule and in the tubular epithelial cells by *in situ* hybridization.
9. My results reported here indicate that vertical transmission of PCV2 does occur and this virus neither replicates in the porcine fetus nor causes abortation and stillborn piglets. The thymus has serious and determinative role in the etiology of immunosystem insufficiency and wasting syndrome („biological thymectomy”).
10. Sequence of the PCV-2 was different, but there were not amino acid changes.
11. I found connection between the replication of the PCV-2 and the histopathological lesions.
12. I diagnosed the porcine inclusion body rhinitis in Hungary for the first time.
13. I investigated the target point of the PCMV and the virus causing disorders..
14. The PCMV infections occurred in three forms: reproductive failure in sows, suddenly death of piglets and rhinitis with clinical signs.
15. The PCMV is widespread among the examined breeds.

Articles

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4. **Z. DEIM**, R. Glavits, F. Szalay, K. Ursu, I. Biksi: Porcine bursitis: Cystic adventitial disease of the vena saphena, Vet. Rec., accepted for publication.
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6. **Z. DEIM**, L. Dencso, R. Glavits, I. Biksi: A role of porcine circovirus type 2 in aborted fetuses and stillborn piglets, Vet. Rec., sent for publication

Presentation

1. **DEIM ZOLTÁN**, DENCŐ LÁSZLÓ, BIKSI IMRE, RÁCZNÉ M. ÁGNES, GLÁVITS RÓBERT. Újabb ismeretek a sertések dermatitis nephropathia szindrómájának kóroktanához és kórfejlődéséhez. Köves-napok, Nyíregyháza, 2005
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3. **DEIM ZOLTÁN**, DENCŐ LÁSZLÓ, BIKSI IMRE, RÁCZNÉ M. ÁGNES, GLÁVITS RÓBERT. A sertés cirkovírus (pcv-2) oktani szerepének vizsgálata a kocák egyes szaporodási zavaraiiban és a magzatok károsodásában. MTA akadémiai beszámolók, Budapest, 2005
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