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Epidemiological examination of porcine circoviruses

Thesis of PhD dissertation

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1. Introduction

Two types of porcine circoviruses (PCV) are currently recognized: the non-pathogenic PCV1 and the pathogenic PCV2, the later associated with several clinical conditions, referred to as porcine circovirus diseases (PCVDs). PCV2 is divided into 2 genotypes. The most characteristic difference between them is the genome length: the longer sequence is designated PCV2A and the shorter PCV2B genotype. During the first recognized cases of PCV2 infections viruses belonging mainly into genotype PCV2A were detected, but by 2003 in Western Europe and by 2005 in North-America PCV2B genotype had become more frequent. The first PMWS case in Hungary was described in 1999. At the beginning of the Hungarian epidemic also PCV2A genomes were detected more frequently, until 2003 when PCV2B started to take over. PCV2 is responsible for severe economic losses of the swine industry. Among PCVDs, the most frequently diagnosed is the postweaning multisystemic wasting syndrome (PMWS). The first description of PMWS was made in Canada, in 1991, but according to retrospective studies, PCV2 itself has already been present in swine samples from the 1970-ies and ‘80-ies. By today PCV2 has spread worldwide. The infection is connected with the following diseases: porcine respiratory disease complex, (PRDC), porcine dermatitis nephrophathy, (PDNS), foetal myocarditis and reproductive disorders. Shortly after PMWS appeared in North

America it was also diagnosed in countries of Europe and Asia. Besides PMWS, the mostly respiratory condition, PRDC also appeared in North America and has become more frequent than the wasting form. On the other hand PMWS is still dominant in Europe mainly in the central and eastern parts. At present the epidemiological situation is not uniform, the virus infection had become enzootic in the west, while in countries of the eastern part of Europe the virus is still spreading. The differences are also reflected in the clinical pictures observed in different geographic areas. PCV2 is also known to be present in wild boars, seriously limiting the possibilities of eradication programs and representing a constant threat for the domestic pig industry.

2. Aims of the study

The main goal of the research was to collect new information about the epidemiology of PCV2 and it was carried out through the following studies:

1. Survey of PCV2 prevalence in the Hungarian wild boar population.
2. Clarify the epidemiologic situation in Central Europe.
3. Study of the possible role of rodents in the maintenance and spreading of the virus.
4. Generation and characterization of a PCV strain suitable for efficient vaccine development.

3. Materials and methods

3.1. Origin of the samples

Organ samples of wild boar were collected at slaughterhouses where killed game is regularly processed, or collected by the Debrecen Institute of the Central Veterinary Institute, during the period of 2002 and 2003.

The farm where the PCV2 associated diseases (PMWS and PDNS) were first recognized in Romania is located in the north–west region of Transylvania, and has approximately 30000 animals. Lymph nodes, kidneys, lungs, portions of skin and serum samples were collected for PCR, serological and immunhistological examinations.

During 2007, organ samples were tested for the survey of the epidemiological status of PCV2 in Central Europe. These samples were collected from Slovakia, Croatia, Czech Republic, Poland, Romania and Hungary.

3.2. Extraction of PCV DNA

DNA was extracted using the Chelex 100® Molecular Biology Grade Resin (Biorad) as recommended by the manufacturer.

In the mouse experiments, the virus DNA was extracted from

faeces and urine by QIAGENE QIAamp® DNA Stool Mini kit, as recommended by the manufacturer.

3.3 DNA amplification and sequencing

Genomic circovirus DNA was amplified by polymerase chain reaction using primer pairs specific for PCV1, PCV2 only or both for PCV1 and PCV2. The primers were designed according to the database of GenBank and our earlier unpublished data, by appropriate computer programs (Primer2, Scientific and Educational Software, and the Oligo6 program, Molecular Biology Insights Inc.).

PCR for sequencing was performed by the Big Dye Kit (Applied Biosystems, USA), and the results were collected by an ABI310 automatic sequencing instrument at the Genodia Molecular Diagnostic Inc. (Budapest) and the Biomi Inc. (Gödöllő).

The BioEdit version 5.0.6. was used to evaluate the results and to construct whole genome sequences. The alignments were carried out with the MEGA version 4.0 program by the Clustal multiple alignment algorithm. Phylogenetic trees were constructed also with the neighbour-joining method of the MEGA version 4.0 software using the ClustalW multiple alignment model with bootstrap values of 1000.

3.4. Animal experiments

The animal experiments were performed according to EU standards using 6-week-old NMRI (Charles River, USA) female mice. Each experimental group of mice contained 2 animals as negative controls, sacrificed on the last day of the particular experiment. After sacrificing the animals, a macroscopic post mortem investigation was performed and samples of mesenteric lymph nodes, spleen, thymus, kidneys and liver were collected from each mouse. The presence of the virus-DNA was detected by PCR. Mice were infected with the ROM1 virus strain (GenBank number: DQ233257). The animals received $5x10^2$ $TCID_{50}$ of ROM1 in 0.5 ml of tissue culture medium.

Pathogenic examination: Four mice were infected by the intraperitoneal route three times, with two weeks interval. Two weeks after the last injection, the mice were sacrificed.

Virus replication: In this experiment 28 mice were inoculated with PCV2 by the intraperitoneal route. Four mice were euthanized every second day on days 2, 4, 6, 8, 10, 12 and 14 post inoculation (p.i.).

Virus transmission 1.: Four mice were infected intraperitoneally only once each. Six days after the injection, four PCV negative contact mice were mixed with infected mice. After 24 days p.i., two infected and two contact mice were sacrificed. After 28 days p.i., two contact mice were sacrificed again. After 42 days p.i., the last two infected

mice were killed.

Virus transmission 2.: Twenty-four mice were divided into 2 groups (A and B); mice in group A (n=18) were inoculated by the oral route with 100µl of $1x10^6$ $TCID_{50}$/ml ROM1 strain and mice in group B (n=6) were left un-inoculated until day 12 p.i., when they were mixed with group A. From the orally inoculated group 6-6 mice were sacrificed on days 12, 19, 26 p.i., and 3-3 from the in-contact mice on days 27 and 30. Feces and urine samples were also collected daily.

4. Results

4.1. Analysis of PCV2 sequences from Hungarian wild boars, between 2002-2003

Approximately two thousand samples were collected and grouped throughout the study according to the temporal and spatial data provided. PCV2 was detected in 20.5% of all the examined animals. According to partial sequence data the PCV2 genomes were divided into 7 groups (WB-H1-7). Selected members of these groups were sequenced completely. Three of the groups had genomes of 1767 bp in length (WB-H1, WB-H5 and WB-H6), and four of them were 1768 bp long (WB-H2, WB-H3, WB-H4 and WB-H7). No characteristics were observed in the countrywide distribution of these groups,

representatives of each group could be detected regardless of the geographical origin. The nucleotide and predicted amino acid sequences of ORF1 and ORF2 of the seven wild boar PCV2 variants were aligned with selected complete PCV2 genomes available in the GenBank and with all of the sequences reported for Hungarian domestic pigs. The predicted corresponding amino acid sequences were also compared. Based on the full-genome alignments some PCV2 variants of wild boar origin were similar to those detected earlier in the Hungarian swine herds. Similar observations were made when comparing the predicted ORF2 amino acid sequences of the same viruses. The comparative analysis of the ORF1 amino acids generated a different dendrogram. The ORF1 amino acid sequences of WB-H4 and WB-H5 were 100% identical and similarly the WB-H2 and WB-H7 sequences were also identical to each other. When looking at the ORF2 sequences 100% identity could be seen between WB-H3 and WB-H4, and also between WB-H1 and WB-H6, so for example the WB-H4 PCV2 virus is identical to the WB-H3 genome on the ORF2 amino acid level, but at the same time completely identical to theWB-H5 genome on the ORF1 amino acid level. These observations were confirmed by comparisons of the ORF1 and ORF2 sequences on the DNA level. Only 0.4% (3 nucleotides) difference was shown for ORF2 sequences of WB-H3 and WB-H4, whereas these genome segments of WB-H1 and WB-H6 were 100% identical.

4.2. Analysis of PCV1 sequences

According to partial sequence analysis, one sample was chosen containing PCV1 (WB-H8). The WB-H8 genome was completely amplified, generating overlapping double stranded DNA fragments for sequencing. The genome contained 1759 bases like most PCV1 strains except a PK-15 derived virus. The WB-H8 genome was compared with other PCV1 sequences from GenBank. The results showed that the differences between the cell culture derived PCV1 sequences and the PCV1 genome from wild boar were not higher than the differences among the non-wild boar genomes.

4.3. Detection of PCV2 in Romania

The firs PCVD cases were recognized in 2002 in a Romanian pig farm. Complete genome sequences were determined both from PMWS and PDNS cases. The PCV2 genomes present in the two syndromes were 100% identical and belonged into the PCV2B genotype (ROM1, GenBank number: 233257). ROM1 was compared with the previously examined sequences and it showed highest similarity with PCV2 sequences of Hungarian, Austrian, French domestic pig and Hungarian wild boar origin.

4.4. Comparison of PCV2 sequences of Central Europe between 2006-2007

PCV2 sequences from Central Europe were examined in 2007. The samples originated from Hungary, Slovakia, Czech Republic, Romania, Croatia and Poland. The PCV2 sequences from these samples were determined and compared with full genome and ORF2 sequences from GenBank, available before 31 of December in 2007. Among the 37 PCV2 positive samples 34 belonged into the PCV2B genotype, and only two Hungarian and one Croatian sequence belonged into the PCV2A genotype. According to complete genome analysis, the PCV2A genotype was divided into 5 groups (A/1, A/2, A/3, A/4, A/5) and the PCV2B genotype was divided into 7 groups (B/1, B/2, B/3, B/4, B/5, B/6, B/7). Among the 411 complete genomes, 293 sequences belonged to the PCV2B genotype, from the 293 sequences, 149 sequences belonged into the B/1 group, among them were 30 newly identified sequences from the Central European region. One Hungarian pig and two wild boar sequences isolated earlier also belonged into the B/1 group. Among the sequences from the PCV2A genotype, one of the Croatian viruses was similar with a Chinese sequence from 2007, which belonged into the PCV2B genotype and with other Chinese sequences from 2007, also members of the PCV2B genotype, but these sequences were not classified into any one of the groups.

On the phylogenetic tree based on predicted ORF2 amino acid sequences, the viruses belonging to the PCV2A genotype were grouped similar to the picture based on the complete genome, but the sequences in the PCV2B genotype were mixed compared with the complete genome.

4.5. Results of mouse infection experiments

Mice were infected with the ROM1 PCV2 strain. No clinical or pathological changes due to PCV2 infection were detected in the sacrificed animals.

Pathogenic examination: No clinical or pathological changes due to PCV2 infection were detected in the sacrificed mice 14 days after the last injection. PCV2-DNA was detectable in the thymus, spleen, kidneys and in liver according to PCR examination.

Virus replication: The virus was detected first at day 2 post infection (p.i.) in the lymph nodes, thymus and the liver. From day 4 p.i. the virus could not be detected in the tissues. Between day 6 and 12 p.i. PCV2 reappeared in the lymph nodes, spleen, kidneys and the liver according to PCR examination.

Virus transmission 1.: PCV2-DNA was detectable from all of the intraperitonaelly infected mice. Two contact mice from the four were also positive for PCV2-DNA according to PCR examination. After 42 days p.i., the intraperitoneally infected mice were also positive.

Virus transmission 2.: In this experiment (based on the previous one) sample collections from the orally inoculated mice started on day 12 p.i. Except for the thymus, the virus could be detected in the organs collected from the first sampling day. Eleven mice from the 18 were positive for PCV2-DNA according to PCR examination. The virus was transmitted to the contact mice according to the PCR results. Feces and urine samples were collected daily from the mice infected orally and PCV2 could only be detected on day 14 in the fecal contents and on day 22 from the urine.

5. Discussion

5.1. Analysis of PCV sequences from Hungarian wild boars, between 2002-2003

The first cases of PMWS were seen in 1999 in Hungary and initially, the incidence of the disease was only sporadic, but PCV2 infection spread rapidly within a few months to practically all of the Hungarian pig herds, which by today are considered to be PCV2 infected. Despite the widespread occurrence of PCV2 in domestic pigs worldwide, only limited data are available about the incidence of PCV2 in wild animals, and those are mostly based on serological studies. One of the purposes of this study was to estimate the

prevalence of PCV in Hungarian wild boars, using samples collected between 2002 and 2003. More than 2000 organ samples were collected from different areas of Hungary. PCV2 could be detected in 20,5 % of the samples, and the proportion of the two genotypes was close to even. Wild boar specific sequences could not be detected. Based on sequencing data the possibility of genomic recombination was raised and later it was confirmed by other researchers. We demonstrated that PCV1 was also present in wild boars and were the first ones to determine the complete nucleotide sequence of a PCV1 from an organ sample. The sequence differences of the wild boar PCV1 and PCV1 from *in vitro* sources was not higher than that among the already known PCV1 isolates.

5.2. Detection of PCV2 in Romania

During our studies we participated in the first description of PCV2 caused diseases in Romania, both PMWS and PDNS. In these cases, the pathological and histological findings were identical with those described in the literature. Complete PCV genome sequences were determined both from PMWS and PDNS affected pigs and they proved to be identical. ROM1 PCV2 was mostly similar to the Hungarian, Austrian, French PCV2 sequences and to sequences from Hungarian wild boars, but the origin of the virus could not be determined.

5.3. Comparison of PCV2 sequences of Central Europe between 2006-2007

To clarify the molecular background of PCV2 epidemiology in the Central European region, PCV2 sequences of this area were studied. Complete PCV2 genomes were determined from Hungarian, Polish, Slovak, Czech, Croatian and Romanian swine samples. The sequences were compared with other PCV2 sequences deposited in the GenBank before 31 of December, in 2007. The oldest available sequences of the region, from 2003, were available from Hungary and Austria, and PCV2A was the dominant type of the time. According to our findings, similarly to the situation in Western Europe, PCV2B had also become dominant in Central Europe although with a few years delay by the year 2007. A similar tendency is observed on a world-wide scale, the shorter genotype is spreading faster than the longer one, and an epidemiologically uniform picture is becoming evident.

5.4. Infection of mice with PCV2

An efficient prevention and control of PCVD requires information of the host range of the virus, whether PCV2 can be maintained and spread by animal species other than swine. Mouse as one of the possible vector species, was studies and it was demonstrated, that PCV2 is indeed capable of infecting mice, the virus can replicate and it

can be shed to be transmitted to other animals, possibly to swine as well. According to the results of our experiments it is safe to suppose, that this rodent may play an important role in spreading PCV2.

6. New scientific results

The PCV infection of wild boars was investigated and it was established that, the same viruses are present in domestic pigs and in wild boars, and the epidemiological changes in domestic pigs were also evident in wild boar populations.

Our results were the first ones to indicate the possibility of recombination as part of PCV2 evolution. This finding was later confirmed by researchers in Asia and North-America.

We were the first to determine a PCV1 complete genome from an affected organ, namely from a wild boar.

The first Romanian PMWS and PDNS cases were described and the PCV2 genomes were determined from both syndromes. Both syndrome were caused by genetically identical viruses.

The first Polish, Czech, Romanian and Croatian PCV2 sequences were determined. PCV2B genotype dominance was determined in Central Europe.

We were the first to report that PCV2 is capable of infecting mice through the oral route and to spread the virus, possibly playing an important role in PCV2 epidemiology.

7. Publications

ARTICLES:

CSÁGOLA A., KECSKEMÉTI S., KARDOS G., KISS I., TUBOLY T.: Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. Arch Virol. 2006. 151(3) p: 495-507. (**IF**: 1,850.)

CADAR D., **CSÁGOLA A.**, DÁN Á., DEIM Z., SPÎNU M., MICLĂUŞ V., KÖBÖLKUTI L., CZIRJÁK G., TUBOLY T.: Porcine circovirus type 2 and associated diseases in Romania. Acta Veterinaria Hungarica, 2007. 55(1) p.:151-156. (**IF**: 0,474.)

CSÁGOLA A., KISS I., TUBOLY T.: Detection and analysis of porcine circovirus type 1 in wild boars. Acta Veterinaria Hungarica, 2008. 56(1) p.:139-144. (**IF**: 0,474.)

CSÁGOLA A., CADAR D., TUBOLY T.: Replication and transmission of porcine circovirus type 2 in experimentally inoculated mice. Acta Veterinaria Hungarica, 2008. 56(3) p.: 421-427. (**IF**: 0,474.)

BÁLINT Á., TENK M., DEIM Z., RASMUSSEN T.B., UTTENTHAL Å., **CSÁGOLA A.**, TUBOLY T., FARSANG A., BERG M., BELÁK S.: Development of Primer-Probe Energy Transfer real-time PCR for the detection and quantification of porcine circovirus type 2. Acta Veterinaria Hungarica, közlésre elfogadva. (**IF**: 0,474.)

IVANICS R., **CSÁGOLA A**., TUBOLY T.: DNS vakcinák, egy újabb lehetőség a fertőző betegségek megelőzésére. Magyar Állatorvosok Lapja, 2004. 126. 617-625. (**IF**: 0,108.)

PATENT:

CSÁGOLA A., PÉNZES Z., TUBOLY T.: Novel porcine circovirus type 2B isolate and uses thereof. US Serial No. 61/118,505, the filing date of November 28, 2008.

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