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Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars

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Summary. Porcine circoviruses (PCV) are present in pigs worldwide; they are grouped into two types: PCV1 comprising non-pathogenic viruses and PCV2 responsible for several clinical manifestations. Both types are frequently detected in domestic pigs, the prevalence and role of PCV in wild boars however, is not well studied. During the years 2002–2003 over 2000 organ samples of Hungarian wild boars were collected, grouped and samples from 307 different animals were tested by polymerase chain reaction for the presence of PCV. 35.5% of the wild boars were positive for one or both PCV types and PCV2 was detected in 20.5% of the animals. The PCV2 viruses were divided into 7 groups (WB-H1-7) based on sequencing data and genomes representing these groups were sequenced completely. The wild boar PCV2 groups were distributed evenly in the geographical region, regardless of the time and place of collection. The phylogenetic analysis of the PCV2 sequences of wild boar and domestic pig origin showed the possibility of an epidemiological link between wild boar and domestic pig infections. Interestingly, the complete nucleotide sequence of the viruses and the predicted amino acid sequence of the replication associated protein (ORF1) grouped the viruses similarly, whereas the capsid protein (ORF2) comparisons revealed different relations among the groups, suggesting the possibility of genomic recombination in PCV2.

Introduction

Porcine circovirus (PCV) was first recognized as a contaminant of pig kidney cell cultures [30, 31]. The virus has a single stranded, circular DNA genome of

Note: Nucleotide sequence data reported are available in the GenBank databases under the accession numbers listed in Table 2.

1.76 kilobases and a virion size of approximately 17 nm. PCV was considered to be a non-pathogenic virus until connection between PCV and the postweaning multisystemic wasting syndrome (PMWS) of pigs had been established [7]. Based on antigenic properties, pathogenicity and nucleotide composition PCV can be divided into two groups: non-pathogenic viruses of the PCV1 group and members of the PCV2 group recognized as the causative agents of PMWS [1]. Retrospective studies indicated that PCV2 and PMWS had been present in domestic pigs well before the first reports of the disease [15, 23, 25]. PMWS by now is present worldwide, it was first diagnosed in Hungary in 1999 [11].

PMWS affects mainly weaned, 5- to 12-week-old piglets, and is of increasing importance to the pig industry because of the great economic losses associated with the disease [1, 7]. It is characterized by weight loss, dyspnoea and jaundice, combined with the pathological findings of interstitial pneumonia, enlarged lymph nodes, hepatitis and nephritis [3, 1]. Lymphocyte depletion has also been reported in lymphoid organs of field cases of PMWS [22] indicating that pigs infected with PCV2 may be immune compromised.

Since the identification of PCV2 and its association with PMWS, the virus has also been detected in pigs affected with various other clinical conditions such as the dermatitis and nephropathy syndrome [23, 36], congenital tremors [29], exsudative dermatitis [35] or reproductive disorders [20], but PCV2 can also be detected in pigs without clinical and histological evidence of these conditions suggesting the occurrence of subclinical infections [14].

Serological studies of wild boar samples indicated that PCV1 was present in these animals [32]. PCV2 specific antibodies were also detected in wild pigs [24, 27, 34], and studies showed the presence of PCV2 genomes and PCV2 antigens in captive or free living wild boars with or without the signs of PMWS [8, 34]. A recent study of 32 Slovenian wild boars [33] based on PCR generated partial sequences of the PCV2 capsid gene (ORF2) indicated a 25% positivity of these animals.

The purpose of this study was a large scale survey of Hungarian wild boars for the presence of PCV2, the establishment of a genetic database for PCV2 genomes in these animals and to compare the genomes of PCV2 in wild boars and in domestic pigs, searching for a possible epidemiological link.

Materials and methods

Samples

Organ samples – mostly portions of kidneys, liver, spleen and lymph nodes – of wild boar were collected at slaughterhouses where killed game is regularly processed, or collected by the Debrecen Institute of the Central Veterinary Institute as part of the regular classical swine fever survey, during the period of 2002 and 2003. The samples were kept frozen at −20 °C until processing. The clinical history of the animals was unknown, but the obligatory slaughterhouse examination data did not indicate any of the PCV2 caused diseases. The age of the animals was also not defined but they were mostly adults, mature enough to be hunted. Over two thousand organ samples of different animals were grouped according to their geographical origin and the date of collection and representatives

(samples from 307 different animals) of these groups were processed for PCV2 DNA amplification.

Extraction of PCV DNA

DNA was extracted using the Chelex 100® Molecular Biology Grade Resin (Biorad) as recommended by the manufacturer. Briefly, approximately 0.1–0.2 g of the tissue was cut into pieces and mixed with 500 µl sterile distilled water, 0.1 g Chelex 100® was added, mixed well and incubated at 65 °C for 20 min. After incubation the samples were boiled for 10 min, chilled on ice for 5 min and centrifuged at 5000 g for 5 min in a microcentrifuge. The supernatant was collected, and frozen to −20 °C until used in the amplification assays.

PCV DNA amplification

Genomic circovirus DNA was amplified by polymerase chain reaction using primer pairs specific for PCV2 only or both for PCV1 and PCV2. The primers (Table 1) were synthesized

Table 1. The specifics of the primers used for PCR and sequencing in this study. The primer used for sequencing only is italicized

Name	Sequence	Location (nt)	Specificity
CBB1	5′-GCT GCC ACA TCGAGAA-3′	289–305	PCV1,2
CBB2	5′-CGC ACC TTC GGA TAT AC-3′	1584–1568	PCV2
CBB3	5′-GCT CTC CAA CAA GGT ACT-3′	437–420	PCV2
PCIIR*	5′-ACA GCA GTT GAG GAG TAC C-3′	853–835	PCV1,2
CSZ1	*5′-ACT ACT CCT CCC GCCATA C-3′*	*1307–1289*	*PCV2*
CSZ2	5′-GGA GGA GTA GTT TAC ATA GGG G-3′	1297–1318	PCV2

*Molnár et al. [17]

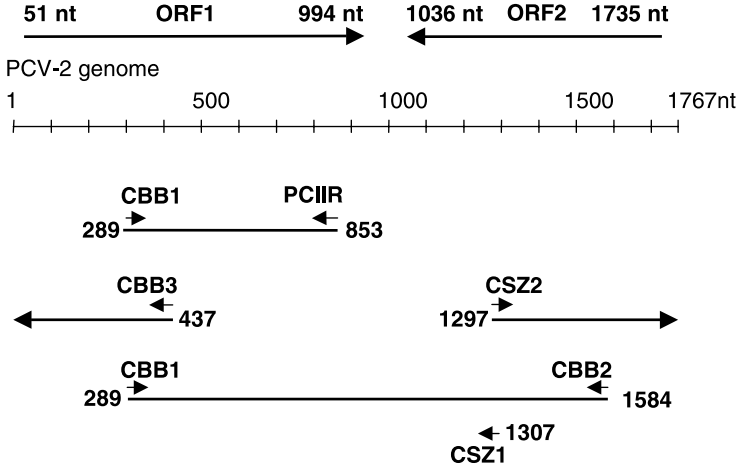

Fig. 1. Linear representation of the PCV2 genome. The arrows on top show the position and direction of the two main open reading frames (ORF1: replicase encoding gene, ORF2: capsid protein encoding gene) of the virus. Lines and arrows below the genome show the exact nucleotide positions of the primers used for the amplification of the indicated products

according to published data [17] or designed using appropriate computer softwares (Primer2, Scientific and Educational Software and the Oligo6 program, Molecular Biology Insights Inc.).

Primers CBB1 and PCIIR were used for the detection of PCV genomes in the tissue samples. Overlapping double stranded DNA fragments of entire PCV genomes were generated by the CBB1–CBB2 and the CBB3–CSZ2 primer pairs. The amplicons were analyzed by partial sequencing using the CBB1 and CBB2 primers and based on the sequence data the samples were grouped again. The relative position and the orientation of the primers together with the size of the amplicons are shown in Fig. 1.

The DNA amplification was performed in a TGradient Thermocycler (Biometra) using 1 μl of each sample in the presence of 3 μl $MgCl_2$(25 mmol), 1 μl of each dNTP (1 mmol), 0.5 μl of the primers (25 pmol) and 1 unit of Taq DNA polymerase (Fermentas) in a final volume of 50 μl, under the following conditions: preheating at 95 °C for 5 min, followed by

Table 2. List of the PCV genome sequences used for phylogenetic comparisons

Name	GenBank	Original name	Reference
AUT3	AY424403	AUT3	Exel et al., 2003 unpublished
AUT5	AY424405	AUT5	Exel et al., 2003 unpublished
CAN1	AF109398	–	Hamel et al. [9]
CAN2	AF118097	IAF-4370	Ouardani et al. [21]
CHI	AF381176	HR	Lu et al., 2001 unpublished
FRA1	AY322000	Fd1	de Boisseson et al. [6]
FRA2	AY321984	Fd3	de Boisseson et al. [6]
GER1	AF201305	GER1	Mankertz et al. [16]
GER3	AF201307	GER3	Mankertz et al. [16]
GER4	AY713470	–	Knell et al., 2004 unpublished
IRL2	CQ768114	–	Allan et al., 2004 unpublished
IRL3	CQ768115	–	Allan et al., 2004 unpublished
JAP1	AB072301	PCV33	Imai et al., 2001 unpublished
SPA1	AF201308	SPA1	Mankertz et al. [16]
TAI1	AY146991	Pingtung-1	Liao et al., 2002 unpublished
TAI2	AF465211	SC	Wang et al., 2002 unpublished
USA	AJ223185	ISU-31	Morozov et al. [18]
HUN1	AY256460	375	Dán et al. [5]
HUN2	AY256456	224	Dán et al. [5]
HUN3	AY256458	326	Dán et al. [5]
HUN4	AY256457	304	Dán et al. [5]
HUN5	AY256459	336	Dán et al. [5]
HUN6	AY256455	212	Dán et al. [5]
WB-H-1	AY874163	–	this paper
WB-H-2	AY874164	–	this paper
WB-H-3	AY874165	–	this paper
WB-H-4	AY874166	–	this paper
WB-H-5	AY874167	–	this paper
WB-H-6	AY874168	–	this paper
WB-H-7	AY874169	–	this paper
PCV1	AF071879	–	Niagro et al. [19]

35 cycles of 95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 60 sec and finishing with a single cycle of 72 °C, 7 min and subsequent cooling to 4 °C. For the CBB1 and PCIIR primers 52 °C was used as annealing temperature, and the elongation step was extended to 90 sec and 120 sec for the CSZ2–CBB3 primers and for the CBB1–CBB2 primer pair, respectively. The PCR products were analyzed by electrophoresis in 1% agarose gels (Q-Biogene) in the presence of 0.4 μg/ml ethidium bromide.

Sequence analysis

PCR fragments were purified using the Geneclean^{R} Turbo for PCR kit (Q-Biogene) and sequenced at the Genodia Molecular Diagnostics Laboratory (Budapest, Hungary) with the primers indicated in Table 1, by the Big Dye terminator cycle sequencing using ABI310 automated sequencer. The sequence editing, analysis and prediction of amino acid sequences were conducted using the EditSeq program of the Lasergene package (DNASTAR Inc., Madison, USA). Sequences were compared to PCV2 sequences from the GenBank, shown in Table 2, using a PCV1 sequence as an out-group. The alignments were carried out with the MegAlign program (Lasergene) by clustal multiple alignment algorithm. Phylogenetic trees were constructed with the neighbour-joining method of the MEGA v.2.1 software using the Kimura two-parameter model and 100 bootstrap values to assign confidence values to topology.

Results

Approximately two thousand samples were collected and grouped throughout the study according to the temporal and spatial data provided. 35.5% of the animals proved to be positive for one or both of the PCV genotypes. PCV2 was detected in 57.8% of the positive samples, which is 20.5% of all the examined animals.

According to partial sequence data using the CBB1 and CBB2 primers the PCV2 genomes were divided into 7 groups (WB-H1-7), with 100% identical sequences in each group. 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 sequence homology of the full genomes among the wild boar viruses varied between 94.7 and 99.4%, whereas the predicted amino acid sequence homology for ORF1 (encoding the replication associated protein) was 98.1–100% and 90.1–100% for ORF2.

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. The results of the phylogenetic comparisons are shown in Figs. 2–5.

Based on the full-genome alignments (Fig. 2) some PCV2 variants of wild boar origin were similar to those detected earlier in the Hungarian swine herds [5], namely WB-H1 and WB-H6 grouped with the HUN-1 and HUN-4, whereas

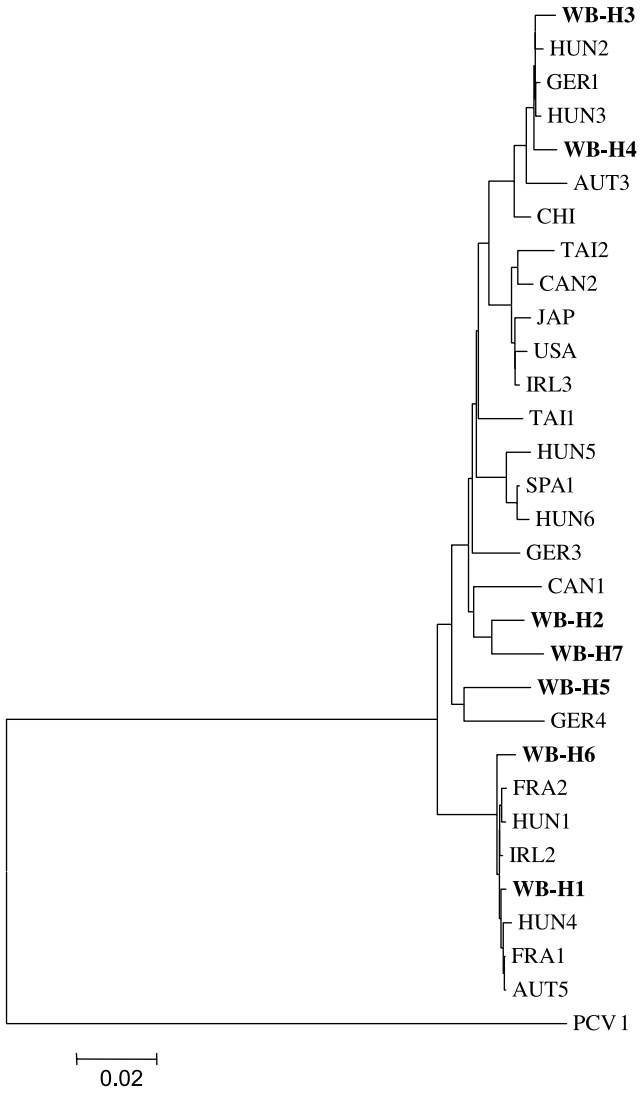

Fig. 2. Comparison of the complete PCV2 genome sequences. Hungarian wild boar genomes are indicated in bold. For GenBank accession numbers see Table 2

WB-H3 and WB-H4 clustered with HUN-2 and HUN-3 strains. WB-H2 and WB-H7 however, showed the closest relationship with a Canadian isolate (CAN1), and the representative genome sequence of the WB-H5 group was similar to a German isolate (GER4) that is the only wild boar strain so far with a genome available in full length in the GenBank database. Similar observations were made when comparing the predicted ORF2 amino acid sequences (Fig. 3) of the same viruses.

The comparative analysis of the ORF1 amino acids (Fig. 4) generated a different dendrogram. Here the Hungarian PCV2 sequences from domestic swine and wild boars grouped differently, HUN-2 and HUN-3 had no close relatives in wild boars. The wild boar sequences WB-H3 and WB-H6 were distinct from

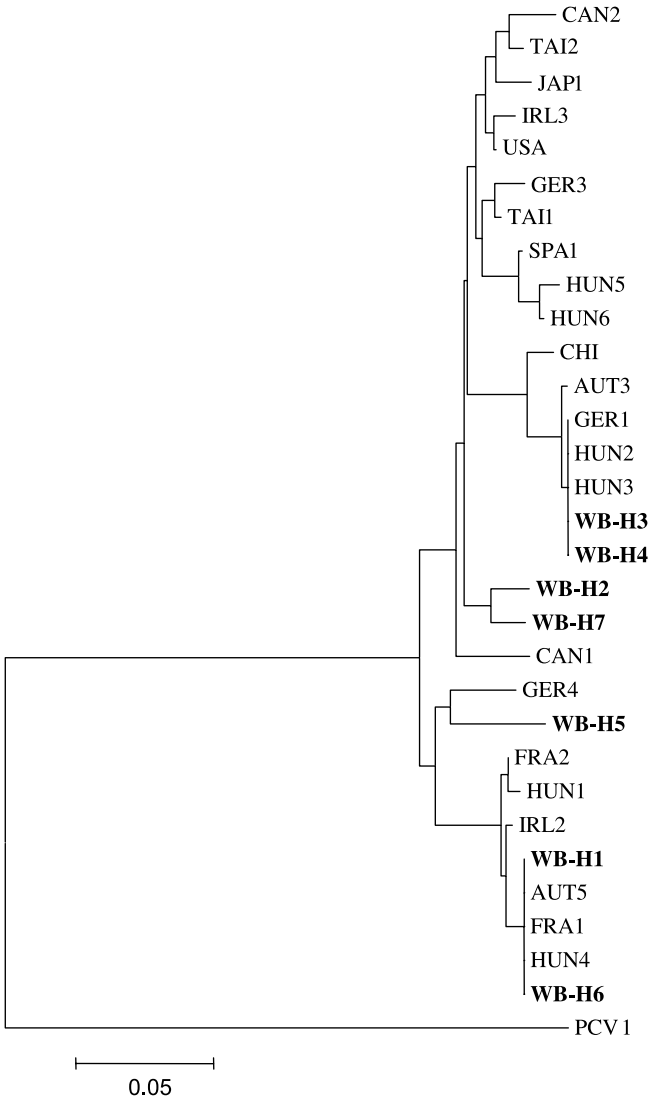

Fig. 3. Phylogenetic tree based on the predicted ORF2 amino acid sequences of selected PCV2 strains. Hungarian wild boar sequences are indicated in bold. GenBank accession numbers are listed in Table 2

other Hungarian sequences, and WB-H2 and WB-H7 had also no domestic swine PCV2 counterpart. WB-H1 grouped with HUN-1 and HUN-4, whereas WB-H4 and WB-H5 showed the highest homology with HUN-5 and HUN-6 from the local PCV2 genomes.

When the analysis was repeated with the WB-H genomes only, the complete nucleotide sequence based tree and the ORF2 amino acid sequence based tree both resulted the same clusters of the wild boar circoviruses (Fig. 5). The predicted ORF1 amino acid sequence based analysis showed, however, a different grouping of the wild boar PCV2 sequences.

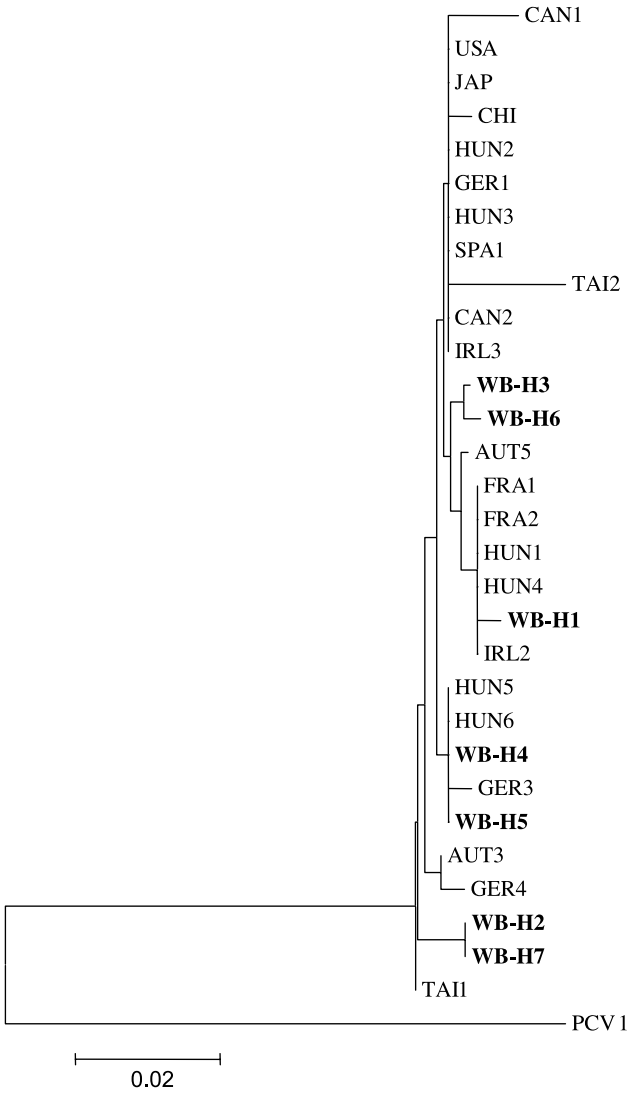

Fig. 4. ORF1 amino acid sequence based phylogenetic tree of selected PCV2 strains. The Hungarian wild boar PCV2 sequences are indicated in bold. GenBank accession numbers are indicated in Table 2

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 the WB-H5 genome on the ORF1 amino acid level (Fig. 5). 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

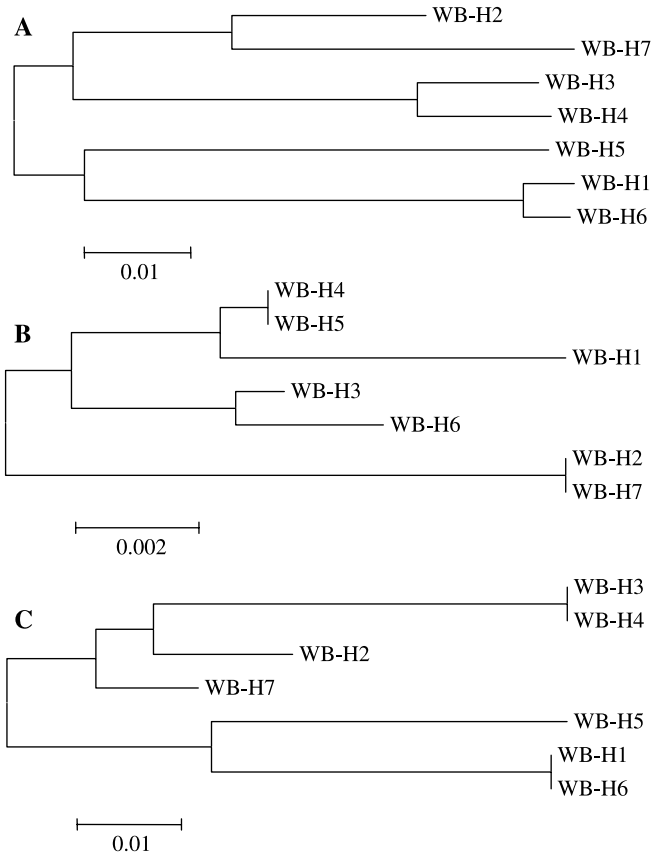

Fig. 5. Unrooted trees based on **A**: the complete genome, **B**: the ORF1 amino acid sequences, **C**: the ORF2 amino acid sequences of PCV2 detected in Hungarian wild boars

genome segments of WB-H1 and WB-H6 were 100% identical. The difference of the ORF1 nucleotide sequence of WB-H4 and WB-H5 was 1.7% and 0.7% for WB-H2 and WB-H7. The amino acid sequences of ORF1 were more conserved than those of ORF2 as indicated by the figures and the bars in Figs. 3 and 4.

Discussion

The first cases of PMWS were seen in 1999 in Hungary [11] and initially, the incidence of the disease was only sporadic [5], 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. Some, however, also provide genetic data suitable for further epidemiological studies. These include the detection of PCV2 genomes in 8 Slovenian wild boars [33], also in one dead wild boar and a crossbred pig in Germany [26] studied in each case both by PCR and sequencing. Mostly partial sequences of wild boar isolates are

available but a full genome sequence originating from Germany was published as a GenBank deposit and it was included in this study as GER4.

The current study proved that both PCV1 and PCV2 were present in the Hungarian wild boars. The incidence of PCV2 in the samples examined was 20.5%, which is slightly lower than that reported for Slovenian wild boars [33] by a similar method. A lower rate of infection in the Hungarian population can not be excluded, however, the difference might also be due to the smaller number of pigs included in the Slovenian study. The seroprevalence of PCV2 reported for Belgian and Spanish feral pig populations [24, 27] was also higher (30–40%) but PCR and serological results cannot be directly compared.

PCV2 of domestic pigs has been described in many countries around the world and the genome sequences of most of the strains detected are available in the GenBank. Complete genomes of PCV2 isolates from different countries including Hungary and the neighboring Austria were aligned with the PCV2 genomes from the Hungarian wild boars. No wild boar specific sequences were detected, although the WB-H5 genome clustered separately, together only with the GER4 wild boar isolate when the full genomes and the ORF2 proteins were aligned. The more conserved ORF1 protein sequence, however, showed that the closest relatives of WB-H5 were two Hungarian domestic pig PCV2 isolates (HUN-5 and HUN-6) and another wild boar PCV2 (WB-H4). The phylogenetic analysis of the ORF2 and the ORF1 sequences revealed higher diversity among ORF2 than among ORF1 sequences (Figs. 3 and 4), indicating that the replicase gene was more conserved than the capsid encoding gene, where selection pressures are more likely to result greater diversity.

The comparison of WB-H1 to WB-H7 genomes based on nucleotide sequences and predicted amino acid sequences of the ORF1 and ORF2 (Fig. 5) resulted phylogenetic trees of different genome clusters depending on the source used in the comparison. The full genome sequences and the ORF2 proteins generated similar clusters of these viruses, whereas the ORF1 based comparisons indicated different relationships. The sequence differences in each case were not high enough to draw further conclusions, but mutations only are unlikely to generate such differences. Whether recombination is responsible for generating viral genomes that group differently when looking at one gene or the other, has to be decided by further studies. Cheung [2] however, proved that the four-stranded tertiary structure of PCV1 allowed for recombination *in vitro*. Evidence for recombination events was also shown for another animal circovirus the beak and feather disease virus [10], where both point mutations and recombinations were found to be responsible for the genetic variation among virus isolates in Southern Africa.

Outbreaks of PMWS have so far been reported mainly in domestic pigs. Reports describing PMWS cases in European wild boar are usually limited to cases when the animals might have had contact with commercial pig farms, where the same virus was detected, or occurred in intensely managed wild boar populations [26, 8, 34]. The rare occurrence of clinical PMWS in wild pigs raises the question whether these animals are less susceptible to the infection, or to the disease itself or losses occur like in domestic animals but consequences such as clinical

signs, pathological lesions or death at the age of weaning remain unnoticed, due to the free roaming nature of these animals. According to estimates, the wild boar population of Hungary had been constantly growing during the past years suggesting that losses, if any, due to clinical PCV2 infections in wild boars are not as devastating as in domestic pigs.

The lower rate of PCV2 infection in free living wild boar, considering the quick spread of the virus in domestic pigs, suggests that those factors that help the spread of the virus in commercial animals such as large populations, lower genetic resistance, weaning as early as possible and vaccinations are not present in wild pigs. Vaccinations by the activation of the immune system especially when adjuvants are used can not only trigger PMWS [4, 12, 13] and contribute to higher doses of virus contaminants in the environment but may also help the direct spread of the virus from pig to pig. Concurrent infections may also be significant factors in triggering PMWS of domestic pigs [28].

Mixed infections of different PCV2 viruses were not detected in this study, but in a number of pigs the PCV1 and PCV2 genomes could be identified parallel. Genetically the PCV2 detected in the Hungarian wild boar population reflected the viruses present in domestic pigs of Hungary or other areas of the world. The wild boar viruses or close relatives were present in domestic pigs and vice versa, suggesting an epidemiological link between free living and farmed animals. It is not known, however, which direction this link transmits virus infections. The almost 100% rate of infection in farmed animals would suggest a virus spread from domestic to domestic pigs and also to free living animals but it is also possible that wild boars serve as natural reservoir for the virus. No other reservoir or vector of the virus had been detected so far that would serve as a common source for both wild and domestic pig infections.

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