

Genetic characterization of porcine circovirus type 2 isolated from different pig-farms in Croatia

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ABSTRACT

Histopathological findings in 25 pig tissue samples, which indicated PCVD (porcine circovirus diseases), were studied. Pig tissue samples originated from 5 different pig-farms in the north-west part of Croatia. Histopathological lesions showed two clinical pictures of the disease: porcine multisystemic wasting syndrome (PMWS) and porcine dermatitis nephropathy syndrome (PDNS). All samples were tested by PCR for the presence of porcine circovirus type 2 (PCV2). Twenty of them were PCV2 positive. PCV2 DNA was quantified by real-time-PCR in all twenty samples with a wide range of PCV2 loads ranging from 10⁵ to 10¹⁰ PCV2 genomes/μL. The highest PCV2 loads were detected in pigs with PMWS lesions, while PDNS affected pigs had lower PCV2 loads. The 20 PCV2 isolates were sequenced and analyzed. Nineteen isolates belonged to PCV2 group 1 and only one to PCV2 group 2. We found no link between genotypes and clinical form of the disease.

Key words: porcine circovirus 2, PCV2, real-time PCR, genotyping

Introduction

Porcine circovirus type 2 (PCV2) causes porcine circovirus associated diseases (PCVD) which represent a threat to the competitiveness of swine farming worldwide through reduced growth and mortality (McKILLEN et al., 2007). PCVD encompasses several syndromes including PMWS (postweaning multisystemic wasting syndrome), PDNS (porcine dermatitis and nephropathy syndrome), reproductive syndrome,

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respiratory syndrome and central nervous system-syndrome in pigs (HARDING, 2004). The factors triggering clinical expression are poorly understood and also the relationships between the genetic groups of PCV2 and their pathogenic effect have not been found (AN et al., 2007). However, viral quantization could be indicative of a clinical disease and PDNS affected animals had lower PCV2 loads than PMWS affected animals (OLVERA et al., 2004).

PCV2 is a member of the *Circoviridae* family. The virion is un-enveloped and icosahedral. The genome of PCV2 is single-stranded circular DNA (ssDNA) (ALLAN et al., 1999). The genome contains two major open reading frames: ORF1 encodes the replicase and ORF2 the capsid protein (MANKERTZ et al., 2004). Phylogenetically, PCV2 can be divided into two major groups (1 and 2) (OLVERA et al., 2007). The most distinct nucleotide differences between two groups are found in the capsid protein gene (33 differences). There is apparently no link between the PCV group and disease status or geographical area (OLVERA et al., 2007). However, some countries have observed a shift from PCV2 group 2 to group 1 (DUPONT et al., 2008; CHEUNG et al., 2007; ELLIS et al., 2006).

The aim of this study was to determine the difference in nucleotide sequences in 20 PCV2 from pigs originated from different pig-farms in the north-west part of Croatia in the period of 2005 and 2006, and also to link the PCV2 load in tissues with PMWS or PDNS symptoms.

Materials and methods

Sample collection. Tissue samples (spleen, lung, lymph nodes) from 25 pig carcasses suspected for PCVD were collected during 2005 and 2006, from 5 pig-farms located in the north-west part of Croatia (Đurđevac, Križevci, Bjelovar, Garešnica and Vrbovec). Seven pigs originated from a pig-farm in Đurđevac, two from Križevci, eight pigs from a farm in Bjelovar, six from Garešnica and two from Vrbovec. All pigs suffered and died with clinical signs indicating PCVD. The most representative symptoms included wasting, dyspnea, palpable lymphadenopathy, diarrhoea and signs like oedema disease. Histopathological lesions also indicated PCV2 infection: hystiocytic infiltration in the lymph nodes, lymphocytic depletion in the spleen, follicular hyperplasia with syncytial cells in the small intestine, membranous glomerulonephritis with hyaline casts in the kidney tubules, and necrotic dermatitis.

DNA extraction and PCR amplification. Total DNA was extracted from spleen, lung and lymph node tissues using the ChargeSwitch gDNA Tissue Kit (Invitrogen, USA), according to the manufacture's instructions. For the amplification of 263 bp of the viral DNA, PCV2 specific oligonucleotide primers (CF8: 5' TAGGTTAGGGCTGTGGCCTT 3' and CR8: 5' CCGCACCTTCGGATATACTG 3') were used (LAROCHELLE et al.,

1999). The final PCR volume was 50 μ L and it was composed of 5 μ L of extracted DNA, 2.5 U *Platinum Taq* polymerase (Invitrogen, USA), 5 μ L 10 \times PCR buffer, 1.5 μ L 50 mM MgCl₂, 20 pmol of each primer and 1 μ L 10 mM dNTPs. After denaturation at 94 °C for 2 minutes, the reaction mixtures were subjected to thermal cycling in a Thermal Cycler 2720 (Applied Biosystems, USA), for 35 ramp cycles each of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. A time delay of 7 min at 72 °C was included prior to a 4 °C soak. Electrophoresis was carried out in a 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide.

Quantitative real-time PCR (Q-PCR). The titre of PCV2 was determined by real-time PCR with PriProET chemistry targeting PCV2-ORF1 as previously described (LADEKJÆR et al., 2002). The equivalent of 100 ng of double-stranded DNA was used as the template in the PCR. A forward primer (5'-GATGATCTACTGAGACTGTGTGA) and a reverse primer (5'-6-FAM-AGAGCTTCTACAGCTGGGACA) were used for the PCR, together with probe specific for PCV2 (5'-TCAGACCCCGTTGGAATGGTACTCCTC-Cy5-3'). The PCR contained a final concentration of 1 \times PCR gold buffer, 2.8 mM MgCl₂, 0.1 mM dNTPs, 0.25 μ M of each primer, 0.5 μ M of PCV2 probe, and 1.25 U of *Amplitaq Gold* DNA-polymerase (Applied Biosystems, Denmark). All reactions were carried out in triplicate in a Rotorgene 3000 (Corbett Research, Denmark). The cycling conditions were one cycle of 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 60 °C for 40 s, and 75 °C for 20 s, followed by a melting curve analysis added for specificity. Fluorescence data collection was performed at each annealing step of the PCR with the increase in Cy5-fluorescence being proportional to the amount of PCV2 DNA in the sample. Two internal positive controls with different but known amounts of PCV2 DNA template were also included. The results are shown as mean of the triplicate reactions.

Genotyping of PCV2 isolates. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) and sequenced on both strands with the sequencing primers SeqPCV2 for (5'-GGATCTTCCAATATCCTATTTG-3') and SeqPCV2 rev (5'-AGAGCTTCTACAGCTGGG-3') for PCR product 1, SeqPCR2 for (5'-CAGCACTGGAGCCCACTC-3') and SeqPCR2 rev (5'-GTGAGGTGTTTCGTCCTCC-3') for PCR product 2, and SeqPCV2 for (5'-GTATTCTGATTACCAGCAATC-3') and SeqPCV2 rev (5'-TACAGAATAAGAAAGTTAAGG-3') for PCR product 3. The PCV2 genomes were assembled using the ChromasPro (version 1.33) program and aligned using the ClustalW method available in the MEGA (version 3.1) program (KUMAR et al., 2004).

Results

From 25 examined samples, 20 were positive for PCV2 by PCR (Fig. 1).

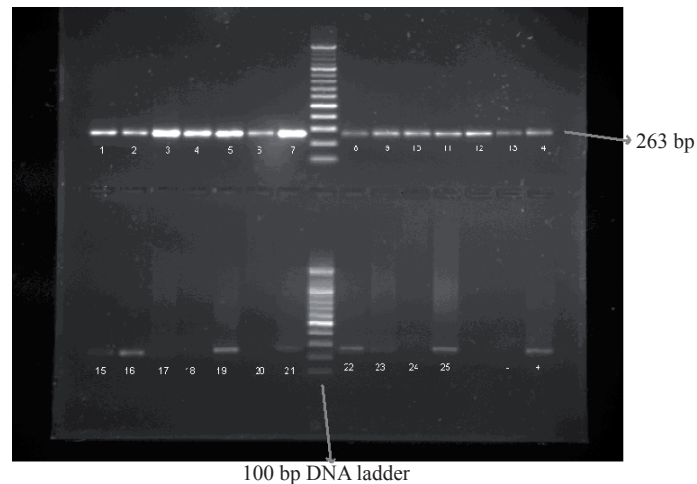


Fig. 1. Detection of porcine circovirus type 2 by PCR, the amplification products were 263 bp long and their length was estimated according to 100 bp DNA ladder (Invitrogen, USA). Twenty-five samples were tested and - indicates negative control, + indicates positive control.

The positive animals were classified regarding PMWS or PDNS histopathological lesions and PCV2 load results through the farms (Table 1).

Table 1. Histopathological lesions and PCV2 loads in examined animals by farms

Pig-farm	Number of positive pigs	PMWS or PDNS lesions	PCV2 genomes/ μ L
Đurdevac	6	4 PMWS+2PDNS	10^7 to 10^{10}
Križevci	1	PDNS	10^5
Bjelovar	7	2PMWS+5PDNS	10^6 to 10^8
Garešnica	5	1PMWS+4PDNS	10^7 to 10^8
Vrbovec	1	PDNS	10^6

The results indicate the presence of both clinical forms of PCV2 disease (PMWS + PDNS) on the pig-farms with greater representation of PDNS.

The PCV2 titre, as determined by realtime PCR, showed high levels of PCV2 DNA in pigs with severe lesions (ranging from 1.93×10^8 to 8.08×10^{10} PCV2 genomes/ μ L) as compared to groups with mild to moderate lesions (ranging from 1.14×10^5 to 7.08×10^8 PCV2 genomes/ μ L). Interestingly, animals with PDNS lesions had a lower viral load than

those with PMWS lesions (Table 1). From eight samples showing $>10^8$ PCV genomes/ μL , six were from pigs with PMWS lesions, and two were from animals with PDNS lesions. From twelve animals having between 10^5 and 10^8 PCV2 genomes/ μL , eleven had PDNS lesions and only one had PMWS lesions.

The PCV2 sequence analysis revealed that 19 out of 20 examined pig samples belonged to PCV2 genotype 1 and only one belonged to PCV2 genotype 2 (Fig. 2). Obviously, there is no link between PCV2 genetic groups and clinical signs of disease (PMWS or PDNS) in the tested pigs. The isolate which belonged to PCV2 genotype 2 was from a pig with PDNS disease.

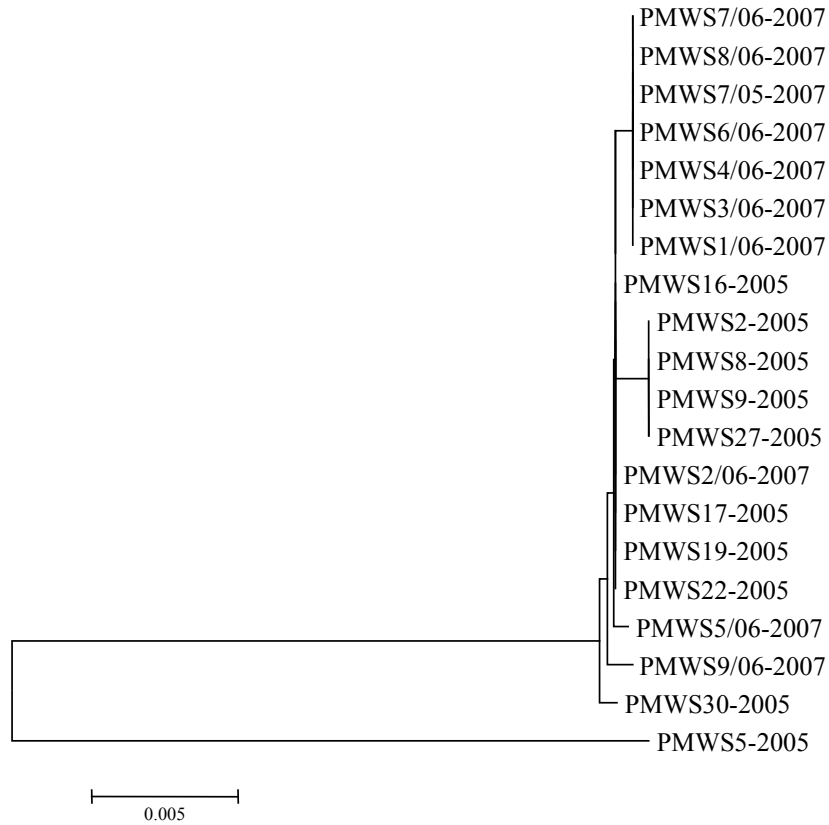


Fig. 2. Genotype analysis of full-length PCV genomes

Discussion

The present study revealed that gross and histological lesions in the pigs with clinical PMWS or PDNS were similar to those previously reported in both naturally acquired and experimentally induced disease (ELLIS et al., 1998; ALLAN et al., 1999; ROSELL et al., 1999).

In all PCV2 infected pigs high amounts of PCV2 genome were detected in the examined tissues. There was a tendency towards higher PCV2 template copy numbers detected by Q-PCR, in tissues from PMWS pigs compared to PDNS pigs.

Although the minor variations in the ORF2 of PCV2 may account for differences in tropism with respect to the host organism, the analyses of PCV2 isolates from PMWS and PDNS cases primarily distinguished minor differences that can be accounted primarily by their geographic origin (MEEHAN et al., 2001).

Clinical PMWS correlated with moderate to severe PMWS-characteristic lymphoid microscopic lesions, and PCV2 viral load increased with the severity of PMWS microscopic lesions (OLVERA et al., 2004). The results obtained in this study also indicate that PCV2 load in tissues could be used for differentiating PMWS from PDNS cases.

PDNS affected pigs, showing mild to moderate microscopic PMWS-like lesions, had low viral loads in concordance with what was found in the PMWS mild lesions category. It is suggested that the presence of high PCV2 loads ($>10^7$ PCV2 genomes/mL of serum) correlates with the presence of PMWS lesions (OLVERA et al., 2004).

The previous studies show that the ORF2 of PCV2, which encodes for the major structural capsid protein, exhibits a higher rate of variation compared to ORF1 (MANKERTZ et al., 1997; LAROCHELLE et al., 2002). There may be a link between capsid protein variation and pathogenicity of PCV2 because of the greater alterations in this protein (CHUNG et al., 2005).

No association between clusters or groupings of PCV2 strains and clinical signs could be found (LAROCHELLE et al., 2002).

The analysis demonstrated that PCV2 group 1 has become dominant with time and that a major shift from the PCV2 group 2 to the PCV2 group 1 occurred in 2003 (DUPONT et al., 2008).

Before 2003, only Canada, USA and Korea/Japan submitted sequences belonging to PCV2 group 2, whereas submissions from the EU and China/Taiwan/Singapore were mixed group 1 and 2 but dominated by group 2. After the shift in 2004, all the countries submitted primarily group 1 sequences (DUPONT et al., 2008).

Our isolates also belong to the PCV2 genotype 1 group.

The global shift confirms similar Danish observations and indicates that group 1 could be a more adapted or even a more pathogenic form of PCV2 (DUPONT et al., 2008).

Recently, phylogenetic-tree analysis has identified two groups (1 and 2) of PCV2 genomes with eight clusters. By analysis, ORF2 genes could not be divided into two subgroups (1A and 1B) of group 1 because ORF1 gene has the key sequence for classification (OLVERA et al., 2007).

The geographical origin of PCV2 strains could not be established (LAROCHELLE et al., 2002; OLVERA et al., 2007).

Interestingly, the PCV2 group 2 strains were found in outbreaks on farms up to 2004, and group 1 has caused continuous outbreaks from then on. From what is known thus far, it appears that the PCV2 strain causing PDNS is not associated with a specific group. No clue exists for any relationship between the groups of PCV2 strains and pathogenic PCV2 isolates from PDNS or PMWS cases (AN et al., 2007).

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SAŽETAK

U ovom su radu izneseni patohistološki rezultati 25 uginulih svinja s pet različitih svinjogojskih farmi na području sjeverozapadne Hrvatske, koje su uginule pod znakovima sindroma kržljivosti odbijene prasadi i sindroma nekrotičnoga dermatitisa. Klinički i patohistološki nalazi upućivali su na cirkovirusnu infekciju svinja. Lančanom reakcijom polimerazom dokazana je virusna DNK svinjskog cirkovirusa tipa 2 u dvadeset tkivnih uzoraka. Ti su uzorci nadalje bili pretraženi lančanom reakcijom polimerazom u stvarnom vremenu kako bi se ustanovila količina virusne DNK. U svim uzorcima ustanovljena je značajna količina virusnoga genoma, koja je iznosila između 105 i 1010 kopija genoma/ μ L. Veća količina virusa dokazana je u onim uzorcima koji su potjecali od svinja uginulih pod znakovima sindroma kržljivosti, a nešto manja količina virusa dokazana je u svinja koje su bolovale od sindroma nekrotičnoga dermatitisa. Svi PCR-proizvodi sekvencirani su kako bi se ustanovila pripadnost određenom genotipu svinjskoga cirkovirusa tipa 2 (SCV-2). Devetnaest izolata od dvadeset sekvenciranih, genotipski pripada skupini 1 SCV-2, a samo jedan izolat skupini 2 SCV-2. Takva genotipska pripadnost podudarna je s podacima iz drugih europskih zemalja, gdje prevladava genotip 1 SCV-2 od 2003. god., a prije toga je dominirao genotip 2 SCV-2 kao etiološki čimbenik cirkovirusnih bolesti svinja.

Ključne riječi: sindrom kržljivosti u odbijene prasadi, sindrom nekrotičnoga dermatitisa, svinjski cirkovirus tip 2, lančana reakcija polimerazom, genotipizacija
