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GENETIC VARIABILITY OF PORCINE CIRCOVIRUS TYPE 2 (PCV2) STRAINS IN CROATIA

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SUMMARY: Porcine circovirus type 2 (PCV2) has been present for the last 2 decades in Croatia, with its highest incidence level in 2004. The clinical features of the disease have altered within this period. Infections until 2008 were mostly accompanied by severe signs such as enteritic and respiratory disorders, erythematous skin lesions that showed a tendency to progress to dermal necrosis accompanied by a high mortality rate of primarily 4 to 16 week-old pigs. Later PCV2 infections were milder and mostly manifested as waste loss and poor growth performance as well as reproductive failure in pregnant sows. In both cases the disease had a direct negative impact on the pig production. Since the infection is continuously present in some regions of Croatia, the heterogeneity of detected PCV2 strains prior to 2008 with strains isolated in 2012 were compared for a better insight in the epidemiological situation. The results of phylogenetic analysis revealed that the viral strains found in 2012 genetically differ from those detected in earlier years. This indicates that new entries into the pig population appeared probably due to pig trade.

***Key words**: PCV2, genetic diversity, clinical manifestation, Croatia.*

INTRODUCTION

PCV2 infections have been recognized through several syndromes such as PMWS (post-weaning multisystemic wasting syndrome), PDNS (dermatitis and nephropathy syndrome), respiratory disease complex, granulomatous enteritis, exudative epidermis, necrotizing lymphadenitis, congenital tremor (Chae, 2005) and reproductive failure (Hansen et al., 2010). The causative agent, porcine circovirus (PCV) is a small, non-enveloped virus with a single-stranded circular DNA of 1.76 kb (Tischer et al., 1982.,

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Crowther et al., 2003) belonging to the genus *Circovirus* within the family *Circoviridae*, along with other animal viruses such as chicken anaemia virus and beak and feather disease virus (Todd et al., 2005). PCV genome contains three open reading frames (ORF), ORF1 encoding a replicase, ORF2 encoding the capsid protein and ORF3 encoding a protein related to cell apoptosis (Mankertz et al., 2004). Two types of PCV have been described, namely PCV1 and PCV2. Their DNA sequence homology is 68 to 76% (Meehan et al., 1998). PCV1 is not pathogenic, and has been detected in cell culture of pig origin used even for vaccine production (Jemeršić et al., 2005), while PCV2 has been defined as the causative agent of pathological syndromes in pigs. Until today, PCV2 isolates are genetically divided into three groups, PCV2a, PCV2b and PCV2c (Opriessnig et al., 2007., Olvera et al., 2007). Boisseson et al. (2004) found that the variations among the PCV2 genomic sequences are mainly due to the variability within ORF2, while ORF1 is highly conserved.

PCV2 infections have been described in Croatia in 1997 and 2004 on large, small and medium size pig farms in several west and eastern Croatian counties (Jemeršić et al., 2004). The morbidity in all studied farms was from 15-30%, while the mortality ranged from 9.3% to 23.8%. Due to high losses in the pig industry, vaccination was introduced to prevent the spread of infection and viral shedding of subclinically infected pigs. From 2008 the clinical signs of disease became less severe in most epidemics recorded, but still remained causes of great losses in the pig industry. Alterations in the predominant PCV2 strains in Croatia or their genetic changes as well as introducing vaccination may have influenced the clinical features of PCV2 infection.

Therefore, we present the sequencing results of the amplified ORF1 of PCV2 strains from 2012 to determine genetic heterogenity of the virus isolates and to carry out the comparison with PCV2 strains collected until 2008.

MATHERIALS AND METHODS

Serum samples of fattening pigs (3-6 months of age) were collected in 2012 from two pig farms in Osijek-Baranja County with a history of PCV2 infection. The samples were pooled (5-10 samples per pool) and kept frozen at -70°C. A total of 10 pools, 5 cointaining samples collected from one PCV2 affected farm (CRO-PCV2-1 to CRO-PCV2-5) and 5 pools from the second PCV2 affected farm (CRO-PCV2-6 to CRO-PCV2-10) were examined for the presence of PCV2 DNA.

An amount of 200 µl of sera was used for viral DNA purification carried out by QIAamp® DNA Mini kit (Qiagen, USA) according to the manufacturer's instructions. For detection of PCV2 DNA a specific primer pair for the amplification of a 360 bp fragment within the ORF1 region was used, according to a previously described protocol by Yang et al., (2003). Reaction mixtures lacking a DNA template were used as negative controls, while a Croatian PCV2-positive sample was used as a positive control. The thermal profile of the amplifications contained an initial denaturation step at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and primer extension at 72°C for 30 sec. The final extension was for 3 min at 72°C.

The amplification products were separated by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized by UV transillumination.

Prior to sequencing PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sent for direct sequencing in both directions to Macrogen Inc., Amsterdam, the Netherlands.

The sequence comparison with the reference strains from the GenBank (Table 1) was performed by algorithm BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Sequencer 4.6. (http://www.genecodes.com, Genes Codes Corporation). Alignment was carried out by ClustalX. For the reconstruction of phylogenetic trees the Neighbor-Joining (NJ) method with Kimura-2 Parameter Model followed by MEGA 5 were used. The clustering stability of the NJ tree was evaluated by bootstrap analysis with 1000 replicates. The PCV2 sequences from Croatia were registered in the GenBank under accession numbers KF498717-KF498720 (CRO_PCV2_1, 3, 4, 6).

Table 1. PCV2 reference sequences used for phylogenetic analysis retrieved from the GenBank.

GenBank Accession No.	Original name	Country
HQ591366	PCV2, isolate 90-08-21	Croatia, 2008
HQ591367	PCV2, isolate 110-08-2	Croatia, 2008
AY256460	PCV2, strain 375	Hungary, 2003
AY424405	PCV2, isolate AUT5	Austria, 2003
HQ591368	PCV2, isolate 126-07-5	Croatia, 2007
HQ591369	PCV2, isolate 147-07-7	Croatia, 2007
HQ591370	PCV2, isolate 161-08-2	Croatia, 2008
HQ591365	PCV2, isolate 70-08-2	Croatia, 2008
AY180397	PCV2, strain Pingtung-5	Taiwan, 2002
AY484410	PCV2, isolate NL-control-4	Netherlands, 2003
AF311296	BFDV	Australia, 2000
AF071879	PCV1	SAD, 1998

RESULTS

The amplification of PCV2 genome regions from samples originating from the two farms with clinical signs of PCV2 (CRO-PCV2-1 to CRO-PCV2-10) resulted in clear PCR products of 360 bp. All strains showed to be members of phylogenetic group 1 or PCV2b group (Figure 1). The seven PCV2 sequences (CRO-PCV2-1, CRO-PCV-2, CRO-PCV2-5, CRO-PCV2-7 to CRO-PCV2-10) were found to be 100% identical among themselves in the ORF1 region. Sequences CRO-PCV2-3 and CRO-PCV2-4 differed in one (0.33%), whereas sequence CRO-PCV2-6 differed in two (0.66%) nucleotides. The obtained phylogenetic clustering shows that the PCV2 isolates differ from the previously published Croatian strains from the year 2007 HQ591368 and HQ591369 in 4.4% of nucleotides whereas when compared to strains from 2008 (HQ591366, HQ591367, HQ591365, HQ591370) in 2.3%, 3.0% and 4.4% (last two) of nucleotides, respectively. The closest genetic similarity of Croatian strains has shown to be with strains detected in Hungary and Austria.

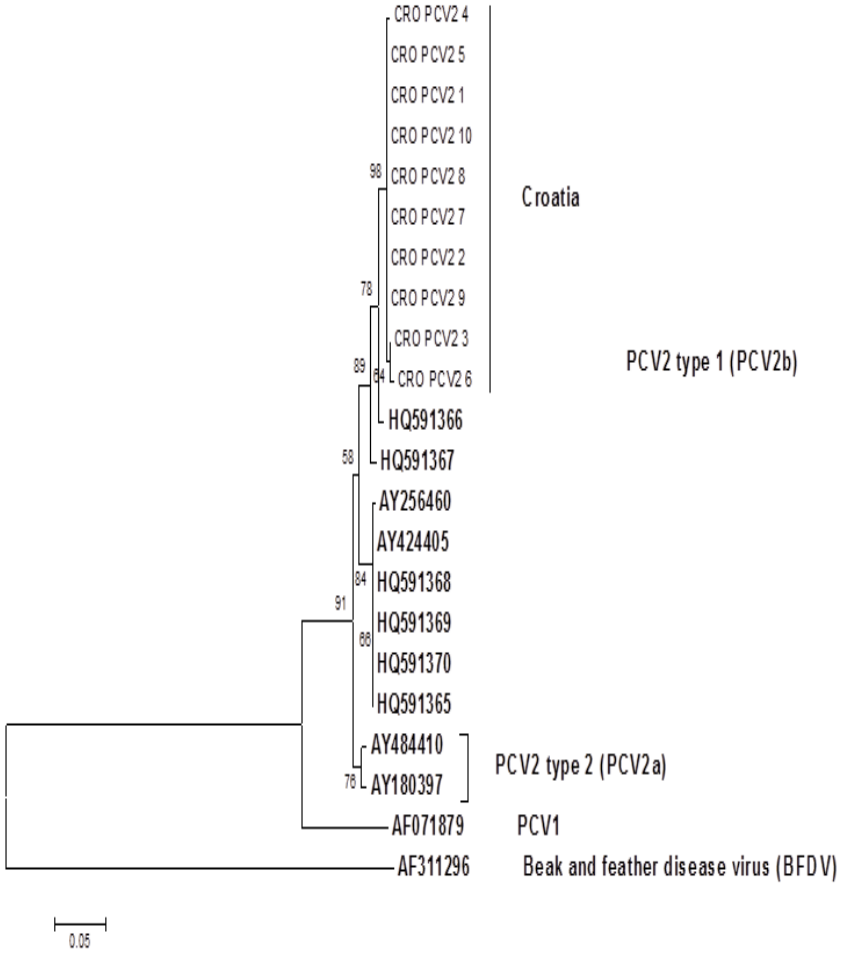

Figure 1. Neighbor-joining phylogenetic tree obtained by the analysis of the partial ORF1 region (297 bp) of Croatian PCV2 samples. Reference sequences included in the analysis are marked in bold. Bootstrap values are presented next to tree nodes. The bar represents 0.05 nucleotide substitution per site.

DISCUSSION AND CONCLUSIONS

Due to the potentially high economic impact PCV2 infections may be considered as one of the most important diseases affecting pig production worldwide (Baekbo et al., 2012). It is thought that PCV2 has been present in pig herds for decades if not centuries before the disease emerged, and the virus slowly mutated into disease-causing forms.

Porcine circovirus type 2 (PCV2) has been detected in pigs with various clinical conditions in different regions of Croatia. The signs that predominated in the affected pigs were skin lesions, loss of weight, respiratory and digestive disorders followed by an increase in the mortality rate. Genetic analyses showed that PCV2 strains from 1997

and 2002 clustered into PCV2a and PCV2b groups, respectively (Jemeršić et al., 2004). Within this study, samples were collected during 2012 from two farms in Croatia with a history of high PCV2 seroprevalence and clinical features of the infection. The 2012 year strains showed clustering into PCV2b group and were more related to those from the year 2002. However, nucleotide differences found between them suggest the appearance of some novel strains. Similar findings were reported and recognised in countries neighbouring Croatia, such as Slovenia (Toplak et al., 2002), Hungary (Kiss et al., 2006), Austria (Schmoll et al., 2002), Serbia (Becskei et al., 2010, Savić et al., 2012), and Italy (Martelli et al., 2009). Interestingly, previously found strains belonging to group PCV2a were not detected in 2012 indicating that this viral group has been eliminated from the tested farms probably as a result of implementing effective biosecurity measures and/or vaccination. Vaccination against PCV2 infection in Croatia was introduced rather late, in 2009/2010. Only commercially available inactivated and subunit vaccines are registered, therefore no chimeric PCV2 was expected to be found during this study, as has been recorded in Canada (Gagnon et al., 2010). However, the milder clinical manifestation of disease recorded from 2008 may be a result of systemic vaccination carried out on farms in combination with the genetic variations found within novel PCV2 strains in Croatia.

Generally, the seroprevalence regarding PCV2 infection is highest in Osijek-Baranja County when compared to other regions in Croatia (Roić et al., 2013) and also contains the highest pig breeding density. Therefore, these were the main reasons to collect the samples from this region.

The detection of novel PCV2 strains and the alteration of egsisting ones suggest that PCV2 is still being introduced into Croatian pig breeding farms, probably due to pig trade. Therefore, apart from vaccination and biosecurity measures, testing of pigs that will be introduced to pig farms would highly contribute in controlling PCV2 infection.

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GENETSKA RAZNOLIKOST SOJEVA CIRKOVIRUSA TIPA 2 (PCV2) U HRVATSKOJ

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Izvod

Cirkovirus tipa 2 (PCV2) je prisutan u svinjogojstvu Republike Hrvatske (RH) tijekom protekla dva desetljeća s najvišim stupnjem pojavnosti u 2004. godini. Klinička slika bolesti je tijekom datog razdoblja promijenjena. Infekcije PCV2 su do 2008. bile većinom praćene teškim znakovima bolesti poput crijevnih i respiratornih poremećaja, crvenila kože s razvojem nekroze okrajnjih djelova tijela svinje, te značajnim povećanjem pomora u prasadi (naročito one starosti od 4. do 16. tjedna). Kasnije infekcije bile su blaže i očitovale su se gubitkom težine, slabim rastom prasadi i reproduktivnim poremećajem u krmača. U oba opisana razdoblja, infekcija je dovodila do značajnih gubitaka i šteta u svinjogojskoj proizvodnji. Obzirom na kontinuiranu pojavu PCV2 infekcije u RH i sa svrhom dobivanja boljeg uvida u stvarnu epizootiološku situaciju u državi, sojevi virusa izdvojeni iz svinja do 2008. uspoređeni su sa sojevima izdvojenima u 2012. Rezultati filogenetske analize ukazuju na različitost sojeva iz 2012. godine u odnosu na ranije izdvojene sojeve. Naši rezultati upućuju na unos ''novih'' PCV2 sojeva u svinjogojsku proizvodnju RH moguće kao posljedica trgovine svinjama.

Ključne rječi: PCV2, genetska raznolikost, klinička slika bolesti, Republika Hrvatska.

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