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PORCINE CIRCOVIRUS TYPE II SCREENING IN FERAL SWINE POPULATION IN UKRAINE

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Summary. Porcine circovirus type 2 (PCV2) is an emergent single-stranded DNA virus found all over the world in domestic pigs and wild boars that causes infectious disease with a great impact on swine productivity. PCV2 has 1.7 kb genome that includes two main genes, which encode replication-related protein (*rep*) and the major structural capsid (*cap*) protein. Both of these genes can be used as target sequences for the primer design for the detection of PCV2 as well as for sequencing of designated regions. We carried out a screening due to the PCV2 circulating among the wild boar population in 10 regions of Ukraine. PCR screening was performed using primer pairs designed on the target sequences of the replicative and capsid genes. According to the results of the research, the presence of genetic material of PCV2 was found in 31.8% of the tested samples. The developed set of primers may be suitable for diagnostics, as well as for the development of specific sites for the purpose of sequencing of PCV2 *cap*-gene due to the obtained DNA samples during epizootic screening

Keywords: PCV2, *rep*-gene, *cap*-gene, epizootic screening, PCR

Introduction. Porcine circovirus type 2 (PCV2) is an emergent single-stranded DNA virus found all over the world in domestic pigs and wild boars that causes infectious disease with a great impact on swine productivity (Segalés et al., 2004). Existing studies showed high prevalence of PCV2 infection in pig farms with severe economic losses worldwide.

PCV2 has 1.7 kb genome that includes two main genes, which encode replication-related protein (*rep*) and the major structural capsid protein (*cap*). Both of these genes can be used as target sequences for the primer design for the detection of PCV2 as well as for sequencing of designated regions (Olvera, Cortey and Segalés, 2007).

Previously using *rep*-gene sequences it has been proposed a set of primers for PCR (Gerilovich et al., 2015). It was used to detect 421 bp amplicon with the further conducting of sequencing and phylogenetic analysis. However, according to other investigations, nowadays the most informative and reliable for sequencing is *cap*-gene due to changes in the sequence of the cap gene which are able to cause variability of the capsid protein and pathogenic properties of PCV2 (Guo et al., 2010; Huang et al., 2013). The *cap*-gene is a more reliable phylogenetic marker for PCV2, since it can reconstruct the similar phylogenetic tree as the complete viral genome (Olvera, Cortey and Segalés, 2007).

Therefore, the **purpose of our studies** was to develop a set of primers based on the capsid gene and to compare its effectiveness in detecting PCV2 during surveillance of samples from feral pigs.

Materials and methods. Archival samples of biological material obtained from wild pigs (organs and blood serum), which were received from hunters during the November–December 2012 hunting season, were used for the research.

DNA extraction from biological material was performed using a commercial 'QIAamp cador Pathogen Mini Kit' ('Qiagen', Germany) according to the manufacturer's manual.

PCR was implemented using 'Maxima Hot Start Green PCR Master Mix' ('Thermo Scientific', USA), primers PCV-2 F/R based on *rep*-gene (PVC-2F CGAAGACGAGCGCAAGAAAATACG, PCV-2R CCAATCACGCTTCTGCATTTTCCC) and primers, obtained in this study.

The PCR products were electrophoresed on 1.5% agarose gel ('Sigma') in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8) using horizontal electrophoresis chamber ('Bio-Rad', USA).

PCV2 *cap*-gene sequences presented at the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov) were used to select specific primers. All sequences were downloaded, linearized at the same point and aligned into database (Benson et al., 2012).

Multiple sequence alignment and target gene selection were performed using Bioedit 7.0.0 and ClustalW module MEGA 6 (Tamura et al., 2013). The design of primers and their subsequent evaluation by PCR quality criteria, intraspecific specificity and range of detection were performed using AmplifiX 1.5.4 and BLAST-online.

Results. In total 107 samples of biological material were obtained from wild boars during 2012 in 10 regions of Ukraine (Poltava, Sumy, Zaporizhzhia, Chernihiv, Chernivtsi, Cherkasy, Kherson, Lviv, Volyn and Luhansk).

The samples were investigated by the classical PCR using a set of primers used the replicative gene as the target sequence. This primer system flanked a 421 bp variable region of the tail *rep*-gene.

According to the PCR results, 34 (31.8%) samples were determined as positive.

The results of the study of samples of biological material from wild boars for the presence of genetic material PCV2 during the hunting season in 2012 are presented in Table 1.

Table 1 — Results of studies of the biological material samples from feral pigs due to the presence of PCV2 DNA

No.	Region	Number of samples	Positive samples	
			Number	%
1	Poltava	15	3	20.0
2	Sumy	8	5	62.5
3	Zaporizhzhia	13	12	92.3
4	Chernihiv	17	5	29.4
5	Chernivtsi	5	2	40.0
6	Cherkasy	10	1	10.0
7	Kherson	1	0	0.0
8	Lviv	13	3	23.0
9	Volyn	13	1	7.7
10	Luhansk	12	2	16.7
Total		107	34	31.8

The largest number of samples was examined from wild boars from Chernihiv region — 17 samples, Poltava — 15 samples, Zaporizhzhia, Lviv, Volyn — 13 samples each. The lowest number (1 sample) was investigated in the Kherson region. Chernivtsi, Sumy, Cherkasy and Luhansk regions — 5, 8, 10, and 12 samples respectively.

Positive specimens for the presence of PCV2 genetic material were detected in all areas except the Kherson region, that was caused, probably, not by the absence of virus circulation, but by the limited number of samples submitted for study (1 sample).

The largest number of positive samples was found in Zaporizhzhia region — 12 positive out of 13 studied, which was 92.3% and in Sumy — 5 positive out of 8 (62.5%). In other regions of Ukraine this indicator was: Chernivtsi region — 2 samples (40.0%), Chernihiv region — 5 (29.4%), Lviv region — 3 (23.0%), Poltava region — 3 (20.0%), Luhansk region — 2 (16.7%), Cherkasy — 1 (10.0%) and Volyn — 1 (7.7%). In general, this indicator averaged 31.8%, which is similar to the data of other researchers both from Ukraine and from other countries.

Thus, we established the presence of genetic material of PCV2 in samples of biological material from wild pigs at the level of 31.8%.

Positive PCV2 DNA samples obtained from screening studies were used to optimize the application protocol for the further primer selection based on *cap*-gene.

The primers were developed based on the target sequence of the PCV2 capsid gene, which resulted in 95 nucleotide sequences of PCV2 downloaded from on-line databases GenBank. In order to identify conserved sites, optimal search parameters were selected and multiple sequencing of the obtained sequences was performed.

Using BioEdit software option conserved regions were analyzed for the presence of primer sequence candidates. The characteristics of the primers were evaluated by quality parameters: length (base pairs), similarity of melting temperatures (stability), the GC/AT ratio at the 3'-ends, avoiding of duplex formation (Rychlik, 1995).

Based on the results of the studies, a primer pair was selected flanking the 798 bp *cap*-gene region (Table 2).

Table 2 — Primer pair for the 798 bp *cap*-gene region amplification

Primer	Sequence (5'→3')
PCV-2 seqF	CCCATGCCCTGAATTTCCA
PCV-2 seqR1	GCGCACTTCTTTCGTTTTC

Bioinformatic analysis of the developed primers showed their compliance with the requirements for their quality and 100% complementarity with the all PCV2 DNA samples.

For validation of the developed set of primers for their ability to identify the PCV2 DNA at the level of diagnostic, 10 samples of nucleic acids, which during the previous screening tests were positive, and 10 negative samples were selected. 798 bp PCV2 DNA amplicons were obtained by PCR using the developed primers according to the following optimized protocol: denaturation primal at 95°C for 5 min; 40 cycles of template denaturation at 95°C for 30 s; 30 s of primer annealing at 55°C; and 45 s of primer extension at 72°C with a final extension cycle at 72°C for 5 min.

The development of amplicons of appropriate length was confirmed by agarose gel electrophoretic analysis.

According to the results of the studies, it was found that the samples that were positive for the detection of genetic material (samples 1–10) by the primer system for the indication of the PCV2 genome by the *rap*-gene showed the same result when developed primer system was used (Table 3).

Table 3 — Comparative results of application by using primer pairs developed on the different targeted gene sequence (*rep* and *cap*)

Result of studies on the presence of an amplicon of appropriate length on the electrophoregrams										
Number of sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
rep-gene (421 bp)	+	+	+	+	+	+	+	+	+	+
cap-gene (798 bp)	+	+	+	+	+	+	+	+	+	+
Number of sample	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
rep-gene (421 bp)	–	–	–	–	–	–	–	–	–	–
cap-gene (798 bp)	–	–	–	–	–	–	–	–	–	–

This indicates that the set of primers developed by us may be suitable for detecting the genetic material of PCV2 in clinical specimens as well as for amplicon synthetize for sequencing purposes.

Conclusions. Screening studies of samples of biological material from wild pigs from 10 regions of Ukraine were conducted.

According to the results of PCR, 107 samples were examined and the presence of genetic material of PCV2 in 34 samples was established, which was 31.8%. PCR screening was performed using primer pairs designed on the base of the target sequences of the replicative and capsid genes.

According to the results of our research, we have demonstrated the effectiveness of our system of primers to the capsid protein gene in terms of its ability to detect the genetic material of PCV2 in the samples.

Prospects for further research. The developed system of primers may be suitable for diagnostics, as well as for the development of specific sites for the purpose of sequencing of PCV2 *cap*-gene due to the obtained DNA samples during epizootic screening.

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