UDC 619:616.98:578.82/.83[PCV2]:578.2'21:636.4(477)

PHYLOGENETIC AND MOLECULAR GENETIC STUDIES OF PORCINE CIRCOVIRUS TYPE II

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Summary. The multiple farms all over the world are affected with porcine circovirus (PCV). This disease has been occurred in Ukraine. This work has been aimed to perform the phylogenetic and molecular genetic studies of porcine circovirus type II that circulate in Ukraine.

This study involves wide spectrum of the molecular techniques, including PCR, RT-PCR and sequencing of variable loci with the next following phylogenetic analysis.

The phylogenetic relationships of porcine circovirus type II that circulate in different geographical regions were studied. The prospect of phylogenetic analysis for genotyping and molecular marking of microorganisms was demonstrated. The porcine circovirus type II indication by the real-time PCR method was described.

Keywords: phylogenetic analysis, real-time PCR, porcine circovirus type II

Introduction. There is a significant problem of porcine circovirus infection in industrial pig farms for a number of Ukraine regions. The results of epizootic studies show its widespread not only in the territory of our country but abroad too (Cano-Manuel et al., 2014). In 1974, there was found a contaminant of the non-pathogenic viral particles in the continuous cell culture line PK-15. It was named the porcine circovirus type I (PCV-1) (Tischer, Rasch and Tochtermann, 1974). The first information about pathogenic porcine circovirus, that later was named as porcine circovirus type II (PCV-2), has been obtained after elicitation of new disease — the porcine multisystemic wasting syndrome (PMWS) in Canada (1991) (Clark and Harding, 1997). The results of porcine infectious diseases researches suggest that PCV-2 takes part in the pathogenesis of nephropathy dermatitis syndrome, proliferative pneumonia, and greasy pig disease, etc. In addition, there is an assumption that PCV-2 is able to initiate the occurrence of violations of reproductive functions in breeding sows (Chae, 2005; Opriessnig, Meng and Halbur, 2007).

The aim of this research was to study the phylogenetic relationships of PCV-2 and to develop the molecular genetic test kits based on method of real-time PCR (RT-PCR) detection of the pathogen in clinical material.

Materials and methods. The programs 4 Mega, ver. 4.0.2, POWER, ver. 1.0 and PhyML ver. 3.0 were used for the phylogenetic analysis (Tamura et al., 2007). For a traditional dendrogram building, based on gene sequences and genomic DNA of PCV-2, the remote-matrix method — a method of nearest neighbors binding (Neighbour joining) — and maximum parsimony method were used (Saitou and Nei, 1987). As a significance test of phylogenetic trees topology, the indexes of repetitions (bootstrap) were used.

For providing of the phylogeographic research of PCV-2, they were selected the completely and partially sequenced genomic DNA and major genes of the pathogen from international databases. All sequences were obtained in the FASTA (*.fasta) or GenBank (*.gb) formats. It allowed to apply the modern molecular biological software (including on-line mode) for multiple sequence alignments and to identify conserved and variable gene fragments as well as insertions, deletions and mutations, construction of dendrogram, and providing of suitable phylogenetic analysis.

The clinical material for research was taken from pigs of different gender and age groups from households of the eastern region of Ukraine. The pathological material had been taken from dead animals or abortive embryos during 2013–2015. Extraction of viral DNA was performed by affinity adsorption method.

The RT-PCR with the application of commercial kit 'Maxima SYBR Green / ROX qPCR' (Thermo Scientific, USA) was used for the derivation of specific DNA-regions. Amplification was performed by the thermocycler DT lite ('DNA technology', Russian Federation). The time and temperature parameters of the amplification are given below:

1st step:	denaturation —	94 °C —	2 min	— 1 cycle
2nd step:	denaturation —	94 °C —	15 sec	40 cycles
	annealing —	58 °C —	1 min	
	synthesis —	72 °C —	20 sec	
	detection —	72 °C —	12 sec	

Results. *Porcine circovirus* genome is presented by a single-strained closed-loop covalent circular DNA

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molecule with length of 1,767 bp. It has two main open reading frames that encode replicase (*rep* gene) and capsid protein of the virus shell (*cap* gene) (Nawagitgul et al., 2000). Using the multiple matching of the genomic DNA sequences and major genes of porcine circoviruses that circulate in different geographical regions and are represented in international databases, it was shown, that the most conservative gene is *rep* (Fig. 1).

There was found only three polymorphic sites (marked as *) in randomly selected fragment of the gene that is 59 bp in length. Analysis of the topology of the phylogenetic tree that was built from gene *rep* sequences, showed the possibility of differentiation of the two main types of PCV — type 1 and type 2, because the PCV-1 representatives belong to the same cluster, and PCV-2 isolate is were separated from the main cluster in a branch (Fig. 2).

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TW-PCV1   ATGCCAAGCAAGAAAAGCGGACCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
AUS-PCV1  ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
USA-PCV1  ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
CH-PCV1   ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
GB-PCV1   ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
HG-PCV1   ATGCCAAGCAAGAAAAGCGGCCCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
CH-PCV2   ATGCCCAGCAAGAAGAACGGACCGCAACCCCATAAGAGGTGGGTGTTCACCCTTAATAA
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Figure 1. Multiple alignment of PCV *rep* gene sequences fragment that was isolated in different countries (USA — United States; CH — China; GB — Great Britain; AUS — Australia; TW — Taiwan; HG — Hungary)

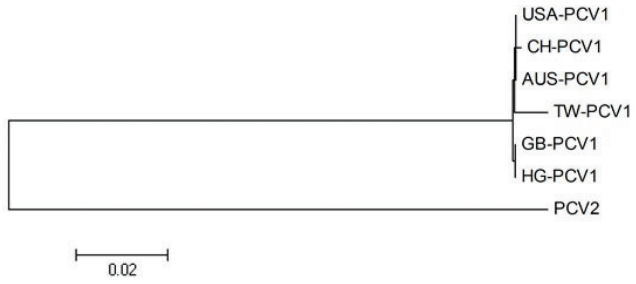

Figure 2. Phylogenetic tree based on rep gene sequences of PCV that isolated in different countries (USA — United States; CH — China; GB — Great Britain; AUS — Australia; TW — Taiwan; HG — Hungary)

However, it is not possible to differentiate genotype of PCV-1 rep gene cluster into isolates due to the high degree of similarity of the analyzed sequences, as it can be seen. The *cap* gene of the porcine circovirus was significantly less conservative: it had been found eleven polymorphic sites (marked *) for randomly selected fragment of the gene that is 59 bp in length (Fig. 3).

Exactly it was the basis for genotyping using the sequences of this gene with taking into account the phylogenetic analysis results.

The results of multiple sequences matching of PCV genes *rep* and *car* confirm to the study data (Olvera, Cortey and Segalés, 2007), where the authors showed that the *rep* gene sequence is identity for circovirus isolates in more than 93%, and identity for *cap* gene sequences is not more than 82%.

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SWE-PCV2  GGGGCTCCAAACCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
USA-PCV2  GGGGGACCAACAAAATCTCTATACCCTTTGAATACTACAGAATAAGAAGAGTTAAGGTT
BG-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
PL-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
SKR-PCV2  GGGGGACCAACAAAATCTCTATACCCTTTGAATACTACAGAATAAGAAAGGTCAAGGTT
BR-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
SB-PCV2   GGGGCTCAAACCCCCGCTCTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
TL-PCV2   GGGGCTCAAACCCCCTCACTGTGCCCTTTGAATACTACAGAATAAGAAAGGTTAAGGTT
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Figure 3. Multiple alignment of PCV-2 *cap* gene sequence fragment that isolated in different countries (USA — United States; JP — Japan; SWE — Sweden; BG — Belgium; PL — Poland; SKR — South Corea; BR — Brazil; SB — Serbia; TL — Thailand)

According to the modern classification, PCV-2 is divided into three major monophyletic groups (Wiederkehr et al., 2009). However, the dendrogram topology that based on the sequences of PCV-2 genomic DNA indicates the impossibility of genotype differentiating of the pathogen, because it is impossible to clearly distinguish clusters that confirm to PCV-2a and PCV-2b genotypes, respectively (Fig. 4).

At the same time, the presence of separate from cluster brunch with isolates of PCV 2a and 2b genotypes allows to admit an assumption about belonging of the strain RVC_UK1 (GB-PCV2 in Fig. 4) to genotype 2c.

According to the modern nomenclature proposed by Segalés et al. (2008), the dependence of PCV-2 to one or another genotype depends on the structure of distinctive motif in the protein capsid — PCV-2a, PCV-2b, and PCV-2c. In its turn, the PCV-2a isolates are second-divided into 5 clusters (2A-2E) and PCV-2b isolates are divided into 3 clusters (1A-1C).

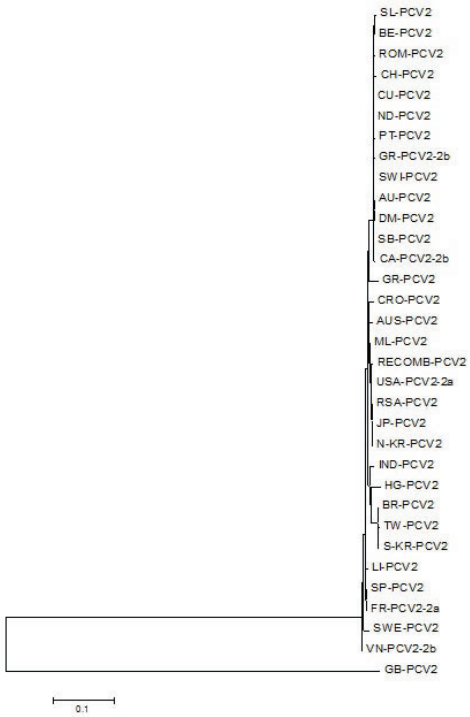

Figure 4. Phylogenetic tree, which bases on gene sequences of PCV-2 DNA isolates that circulate in different countries (SL — Slovakia; BE — Belgium; ROM — Romania; CU — Cuba; ND — Netherlands; PT — Portugal; GR — Germany; SWI — Switzerland; AU — Austria; DM — Denmark; SB — Serbia; CA — Canada; CRO — Croatia; ML — Malaysia; RSA — South Africa; JP — Japan; N-KR — North Korea; IND — Indonesia; BR — Brazil; S-KR — South Korea; LI — Lithuania; SP — Spain; FR — France; SWE — Sweden; VN — Vietnam; USA — United States; CH — China; GB — United Kingdom; AUS — Australia; TW — Taiwan; HG — Hungary)

The topology analysis of the phylogenetic tree (Fig. 5) that bases on the sequences of the *cap* gene from PCV-2 isolates that circulate in different countries demonstrates the existence of at least two subtypes for genotype 2a, two subtypes for genotype 2b, and suggests circulation of PCV-2 genotype 2c in the territory of Argentina and China. Despite the high degree of homology between PCV-1 and PCV-2 genomic DNA, which can reach 82% (Kudin and Prokulevich, 2011), we had proved the possibility of differentiating between these two types of porcine circovirus through phylogenetic analysis based on viral DNA sequences (Fig. 6). The topology of the resulting dendrogram indicates the formation of separate, explicit cluster by PCV-1 isolates. The presence of separate branch for strain PCV-1 IL that circulates in the territory of Taiwan (TW-PCV1, Fig. 5) indicates the genotypic heterogeneity of the pathogen.

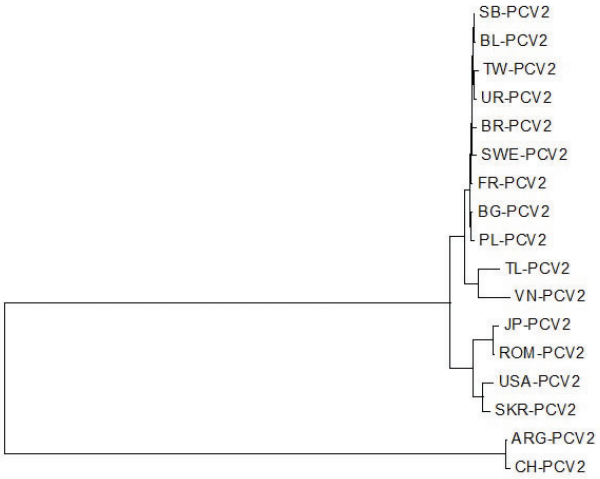

Figure 5. Phylogenetic tree that bases on sequence of PCV-2 *cap* gene from different countries (USA — United States; CH — China; TW — Taiwan; SB — Serbia; BL — Belarus; UR — Uruguay; BR — Brazil; SWE — Sweden; FR — France; BG — Belgium; PL — Poland; TL — Thailand; VN — Vietnam; JP — Japan; ROM — Romania; SKR — South Korea; ARG — Argentina)

The results of phylogenetic analysis that had been performed on the basis of genomic DNA sequences of PCV-1 confirm the possibility of existing, at least, of three genetic groups of the virus due to the existence of three clusters at the linearized phylogenetic tree (Fig. 7).

The results of the topology analysis of the phylogenetic tree that based on the sequences of different circovirus genes has showed the importance of phylogenetic analysis for genotyping and molecular marking of microorganisms once again.

The early diagnosis of viral porcine diseases is necessary for the effective development of pig farming

that is the one of the promising sectors of animal farming in Ukraine at this time. In time, detection of suspected ill animals is an important and necessary step because it affects the efficiency of livestock treatment. The polymerase chain reaction (PCR) in real-time format is one of the reliable and rapid methods for detecting of infectious agents in animals at any stage of the disease, including the early stages. Our results concern the definition of conservative and variable regions in the PCV-2 genome. This fact became the basis for oligonucleotides determining of potential primers for molecular genetic detection of the pathogen.

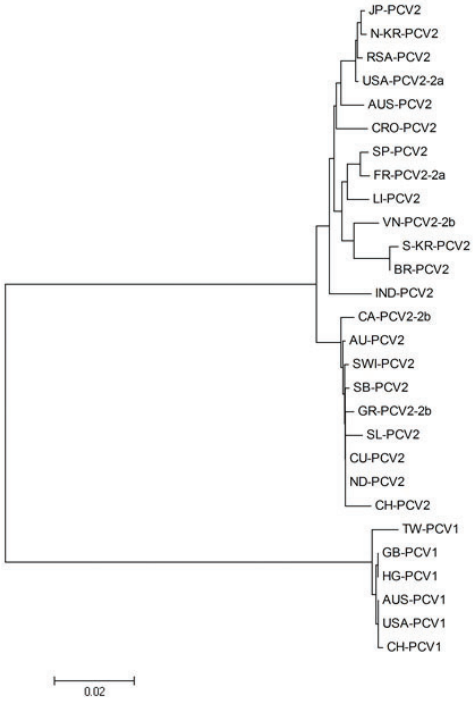

Figure 6. Phylogenetic tree that bases on gene sequences of PCV-1 and PCV-2 DNA isolates that circulate in different countries (USA — United States; CH — China; GB — United Kingdom; AUS — Australia; TW — Taiwan; HG — Hungary)

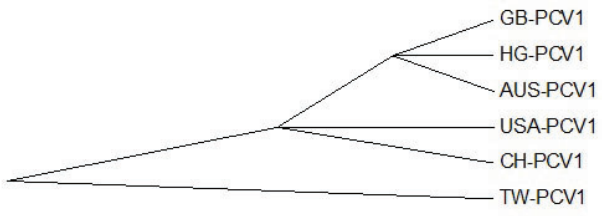

Figure 7. Phylogenetic tree that bases on gene sequences of PCV-1 DNA isolates that circulate in different countries (USA — United States; CH — China; GB — United Kingdom; AUS — Australia; TW — Taiwan; HG — Hungary)

Analysis of the sequence of selected primers for detection of PCV-2 by conventional parameters for quality check (Gerilovych, 2009; Sachse, 2003) has demonstrated their compliance with the applicable requirements and 100% complementarity to the exactly PCV-2 DNA template (Fig. 8).

RT-PCR results of protocol testing established that the optimum temperature for primers annealing for PCV-2 genomic DNA detection is 58 °C, and the threshold cycle equals 25 (Fig. 9, Table 1), that is the useful indicator in the application of this technique (Rebrikov et al., 2009).

For additional monitoring of derivation of specific DNA segments, electrophoretic analysis of the reaction products was conducted. It confirmed the amplification of the specific fragments of PCV-2 genomic DNA that is 97 bp in length (Fig. 10).

Table 1 — The results of real-time PCR analysis of material samples for the presence of genomic DNA specific fragment of PCV-2

Number of sample wells	Identify of test-tube	Threshold cycle Cp, Fam	Result
A1	Sample 1 (TEST)	25,8	+
A2	Зразок 2 (TEST)	25,9	+
A3	Sample 3 (TEST)	26,0	+
A4	Sample 4 (TEST)	25,2	+
A5	Sample 5 (TEST)	25,3	+
A6	Sample 6 (TEST)	25,9	+
A7	Sample 7 (TEST)	25,2	+
A8	Sample 8 (TEST)	27,0	+
B1	Sample 9 (TEST)	27,3	+
B2	Sample 10 (TEST)	26,0	+
B3	K + (TEST)	25,2	+
B4	K - (TEST)		-

Remark: «+» means that sample is positive and contains genetic material of PCV-2

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	ATTACCAGCGCACTTCGG	18	57.80	55.56	4.00
Reverse primer	GGGTCCGCTTCTTCCATT	18	56.93	55.56	3.00

Products on target templates

>LC004734.1 Porcine circovirus-2 genes for capsid protein, replication protein, complete cds, isolate: AR-3

```
product length = 86
Forward primer  1    ATTACCAGCGCACTTCGG  18
Template        769  ..................  786

Reverse primer  1    GGGTCCGCTTCTTCCATT  18
Template        854  ..................  837
```

>KJ956690.1 Porcine circovirus-2 strain HX1, complete genome

```
product length = 86
Forward primer  1    ATTACCAGCGCACTTCGG  18
Template        493  ..................  476

Reverse primer  1    GGGTCCGCTTCTTCCATT  18
Template        408  ..................  425
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>KJ956689.1 Porcine circovirus-2 strain BH6, complete genome

```
product length = 86
Forward primer  1    ATTACCAGCGCACTTCGG  18
Template        493  ..................  476

Reverse primer  1    GGGTCCGCTTCTTCCATT  18
Template        408  ..................  425
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Figure 8. The results the primer system specificity verification using BLAST algorithm

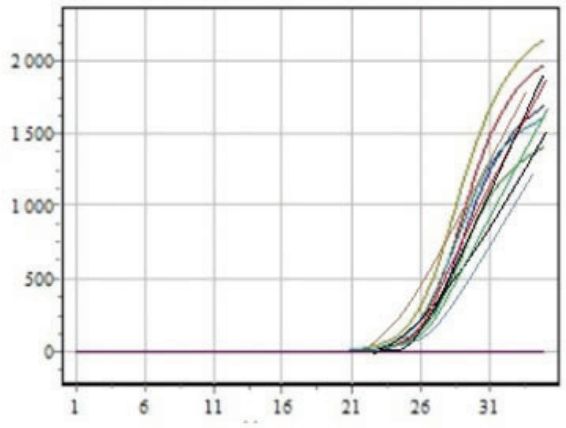

Figure 9. The amplification curves of specific DNA fragment of PCV-2 obtained with the use of SYBR Green intercalating dye

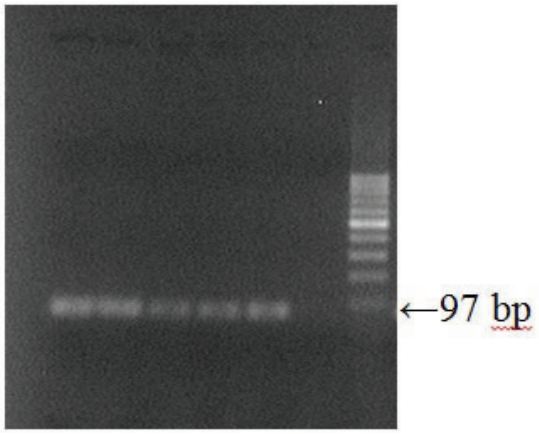

Figure 10. Electrophoretic analysis of the PCR-products obtained by amplification of the specific DNA fragment of PCV-2 with use of SYBR Green fluorescent dye

Conclusions. Thus, it was found that the most conserved gene of PCV-2 is *rep*, which encodes the replicase; and the most variable gene is *cap* that encodes a protein capsid of virus shell. The possibility of the existence of circovirus with 2c genotype for PCV-2 and the existence of at least three genetic groups of porcine circovirus type I were proved. The possibility of differentiation of porcine circoviruses type I and type II was shown due to providing of phylogenetic analysis that based on sequences of viral DNA. The method of PCV-2 indication by real-time PCR was developed.

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