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STUDY OF THE GENETIC VARIABILITY OF THE PORCINE CIRCOVIRUS TYPE 2 IN UKRAINE

Gerilovych A. P., Stegniy B. T., Rudova N. G., Buzun A. I.
National Scientific Center 'Institute of the Experimental and Clinical Veterinary Medicine',
Kharkiv, Ukraine, e-mail: antger2011@gmail.com, antger@vet.kharkov.ua

Summary. Porcine circoviral infection is the economically significant and contagious porcine disease, affecting of the animals from different ages and causing multiple syndromes associated with immunodeficiency. The aim of this work consisted in the study of genetic polymorphism of the *Porcine circovirus* type 2 strains circulating in different farms in Ukraine to determine their genotype.

Study was performed by the molecular (PCR, sequencing) and bioinformatical (multiple alignment, phylogenetic study) methods. Samples were collected using basic methods from the productive pig farms in Eastern Ukraine. *Rep* gene 421 bp region has been used as the matrix for sequencing and phylogenetic analysis.

The 6 characterized isolates were related to 1 and 2 genotypes of PCV-2. They demonstrated different levels of variability in the comparison with strains from European, Asian and American origin, allocated in different countries.

The study of the PCV-2 genetic differences in 421 bp rep gene loci demonstrated relation of isolates to American-origin contaminants of the veterinary preparations — genotype 2, and other field strains were related to European progenitors of genotype 1 (70–93% homology).

Keywords: *Porcine circovirus* type 2, genotype, PCR, phylogenetic analysis.

Introduction. Porcine circoviral infection is a mainly contagious and highly distributed disease of domestic pigs and wild boars. It could be characterized with the development of the system immune deficits, disorders in the digestion and reproductive system, respiratory tract. They could be potentially complicated by the secondary pathogens.

The disease causative agent is *Procine circovirus* type 2, causing Post-wearing multisystem wasting syndrome (PMWS). Circoviral infection is the main disease in commercial and back-yard farms in every country with developed pig-breeding since 2001–2002.

Virus could potentially infect porcine fetus, because it easily migrates via trasplacentary barrier. The colostrum immunity, formatted just after born of the pigs, provide the protection during first 1–2 months of life.

However, after the transferring of the piglets to 2–4 group, when the maternal antibodies level decreases, their own immune system is blocked by circovirus that allows the agents of other viral infections (PRRSS virus, *Porcine parvovirus* etc.), bacterial infections including mycoplasmoses to develop the multiethiological disorders (Anon., 2005; Kovalenko et al., 2005; Orlyankin, Aliper, and Nepoklonov, 2002).

Circoviruses also could affect the adult pigs. It is characterized by development of the reproductive disorders (aborts, dead-birth, birth of the weak piglets etc.). In addition, the literature contains descriptions of infecting the adult animals with the development of the systemic immunodeficiency, which in several cases could be the reason of animal death (Anon., 2005).

Postweaning multisystem syndrome is described in European countries, United States of America, Canada, Brazil, former USSR member states and others. Annual economic losses, caused by PMWS, represent amount of 300 $ millions only in USA (Choi, Chae and Clark, 2000).

Porcine circoviruses are presented by two types — PCV-1 and PCV-2. PCV-1 is apathogenic. PCV-2 is the pathogenic agent, which is more interesting from veterinary medicine point of view. This virus causes the multiple lesions in pigs and piglets, and requires the effective means for diagnostics and surveillance (Choi, Chae and Clark, 2000).

The wide spectrum of the laboratory diagnostics techniques are used for virus detection and characterization in the veterinary practice. The list includes classical virology with isolation of virus in susceptible cells (PK-15), and further identification of the agent using ELISA and in-situ hybridization techniques (Kritas et al., 2007; Allan et al., 2007; Shkayeva et al., 2006; Blotska, 2008).

The different types of ELISA and PCR techniques are used for PCV-infection monitoring. PCR has been recognized as better technique for PMWS surveillance. It is used in classical and real-time modification for detection of virus-keeper animals (Fort et al., 2007; Shang et al., 2008).

Also the multiplex PCR-based protocols has been described to detect circoviruses in complex with other porcine pathogens, including Porcine parvovirus, Aujeszky's disease virus, African swine fever virus and others (Anon., 2007; Kim and Chae, 2001; Lee et al., 2007; Giammarioli et al., 2007).

The method of sequencing of the amplified viral DNA fragments is the one of the most informative techniques for molecular diagnostics and epithology of PMWS. The first genotyping studies were performed by Stevenson et al. (2001) and Allan et al. (1999). These researchers demonstrated presence of two genotypes of PCV-2 based on partial sequences of its genome. The divergence of sequences has been observed in the level of 4–5%. Also they described the correlation between genotype and clinical and pathological signs of the disease.

Two genetic lines (1 and 2) were described after full-length genomic sequencing of the viral DNA. The a and b sublines were detected in each line. Genotype 1 is presented mostly by European PCV-2 isolated, and genotype 2 includes American and Asian viruses. The genetic divergence among genotypes is about 3–5%. The differences are also 0.8–1.3% in genomes of 1a and 1b clades of strains (De Boisseson et al., 2004).

Molecular genetic tests for indication of viral DNA of circoviruses could be characterized as the most effective surveillance and diagnostics. Almost the wide disease spread in Central European

countries determines the necessarily of study of the virus genetic diversity for its characterization from genetic point of view.

The aim of this study was determination of the phylogenetic relations of Ukrainian PCV-2 isolates.

Materials and methods. Amplification of the variable fragment of circoviral DNA in the 421 bp region of *rep* gene was done using PCV-2_F/R primer set, early developed in NSC 'IECVM' [PCV-2 PCR protocol, 2011].

Sequencing was managed by SeqLab GmbH (Göttingen, Germany) and Lohmann Animal Health (Cuxhaven, Germany) by the classical Sanger method.

Sequencing chromatograms were edited using nucleotide sequences manager BioEdit v. 7.1.1.5.

Phylogenetic analysis of the rep gene variable loci was managed by MEGA v. 4.0. software, using neighbor-joining algorithm. Graphical analysis of the constructed trees has been done by TreeView v. 1.6.0.

PCV-2 *rep* gene sequences were published in GenBank sequences database (access numbers: EU275761, EU260051-54, EU252693).

Results of study. In order to establish the molecular characteristics of epizootic PCV-2 strains, detected in farms Ukraine, and study of their phylogenetic relationships with viruses isolated in different regions of the world our studies of DNA the sequencing of PCV-2 in 421 bp area of *rep* gene has been done. This gene is the most representative in terms of molecular epizootiology of porcine circoviral infection. DNA amplification products of PCV-2 *rep* gene 421 bp region were received using PCR. Amplicones has been derived from the material from pig farms in Kharkov, Lugansk, Poltava, Dnipropetrovsk and Belgorod regions. They were purified in agarose gels of impurities nonspecific DNA length. Further, these fragments excised and extracted from the gel method destruction zircon sand and elution of DNA in TE buffer. In order to concentrate DNA elution volume of 50 ± 4 ml dried at 65 °C.

Prepared fragments were assigned names: PCV2 Poltava (amplicon of viral material AF 'Fisun'), PCV2 Lugansk (LLC 'Granum'), PCV2 Lugansk2 (LLC 'Call'), PCV2 Kharkov (AF 'Dubrava'), PCV2 Dnipro ('YUSOLS') and PorBelgorog (FDE Krasnoyaruzkyy SC).

In the first phase were calculated DNA concentration after dilution, samples standardized within 44.2–54.4 mg/cm^3. Sequencing amplification primers carried out in the application system development PCV_1–2 NSC 'IECVM' and standard protocol.

The obtained sequences were analyzed by multiple alignments technique. As a result, the presence of three isolates clades in Ukrainian isolates of porcine circovirus type 2.

First clade, which included isolates PCV-2 Poltava and PCV-2 Dnipro compared to isolate k43 °C (for comparison matrix proposed Gagnon CA) had three mismatches: C → G at locus 308 bp, A → G — → T and 363 G — 365 bp. Second group of viruses included isolates PorBelgorog and PCV-2 Lugansk. These isolates had 11 substitutions in the study area rep gene and deletion of 2 bp size. The third clade isolates: PCV-2 Lugansk2 and PCV-2 Kharkov. They carried 14 nucleotide substitutions in the area rep gene 421 bp in length, but not adequately deletion at positions 198–199 bp by matrix alignment. Character of the replacements in second and third groups diverse loci has been presented 248 and 256 bp (not in group 2), 155, 289, 291, 367, 382 bp (not in group 3) (Fig. 1).

After broadcasting the resulting alignment second clade of viruses had 13 amino acids differences in comparison with the first group (isoleucine (I) → serine (S) at position 52, phenylalanine (F) → S — 53, 55, XX (reading frame shift due to deletions) — valine (V), asparagine (N) — 67, 66, arginine (R) → lysine (K) — 85, R → proline (P) — 103, glycine (Q) → leucine (L) — 107, tryptophan (T) → I 113, V → L — 121, 122, S → N — 123, R → K 130) with almost 140, coded sequence sequenced (Fig. 2).

Group 2 differs from 1st group only in five amino acid positions, and thus only five nucleotide substitutions identified in sequencing were significant (S → F — 55, R → K — 83, L → methionine (M) — 86, V → L — 94, N → S — 123).

Thus, it was established three subgroups virus circulation with a different nucleotide structure relative to each other. This in turn reflected and amino acid sequences of the gene *rep* studied viruses.

After analyzing the genetic variability of the pathogenic PCV-2 DNA, concluded that isolates the second group stimulated the development of respiratory disorders, while two other groups of viruses found in both reproductive and respiratory and when mixed forms of circoviral infection in pigs and piglets.

Analyzing the previous literature described and our own data, it is possible to make the solution that correlation does not always exist between *rep* gene sequence and pathogenic potency of virus (PCV-2). However, analysis of this gene provides us with enough data for study of the phylogenetic connection among the PCVs strains. That was the reason for upcoming phylogenetic study on the next stage of our research.

The Neighbor Joining algorithm in MEGA software has been used for the genetic analysis of PCV-2 strains circulation in pig farms in Ukraine. The rooted phylograms were constructed, and the *rep* gene sequence of canary circovirus has been used as the polarization sequence (Fig. 3).

Isolates PCV2 Poltava and PCV2 Dnipro were situated on the same cluster as early described isolate k43 °C of PCV-2, which is the potential contaminant of the cell lines. This circumstance describes the possibility of cultural origin of the mentioned viruses. It could be possible, that named populations of virus have infected animals via contaminated vaccines and immune-therapeutic preparations.

Other group of viruses on the dendrogram was presented by two subgroups, respectively, presenting the 2nd and 3rd cluster of the analyzed viruses.

The next step of our research was devoted to comparison of the sequences, derived from PCV's DNA samples, belonging to different isolates. This has been done using Kimura's comparison. The calculated data demonstrated the absence of the divergence in the 1st group of viruses, inside the 2nd group is was 1.7%, and for 3rd group – 0.2%. The distances between 1st and 2nd groups were 0.032–0.037, and the distances between 2nd and 3rd — 0.017–0.034, and 0.037–0.037 — between groups 1 and 3 respectively (Fig. 4).

The analysis of the comparison of neutrality evolution indexes by the Nei-Gojobori method demonstrated the high level means of the p-distances among viral groups (over 1.2). The neutrality test by Tajima method allowed to describe 19 segregation sites with segregation indexes (pS) 0.045346, conservatively index (pi) was 0.022730. Pairwise and evolutional indexes demonstrated the likelihood of the analyzed viruses in the frames of the *rep* gene analysis (Fig. 5).

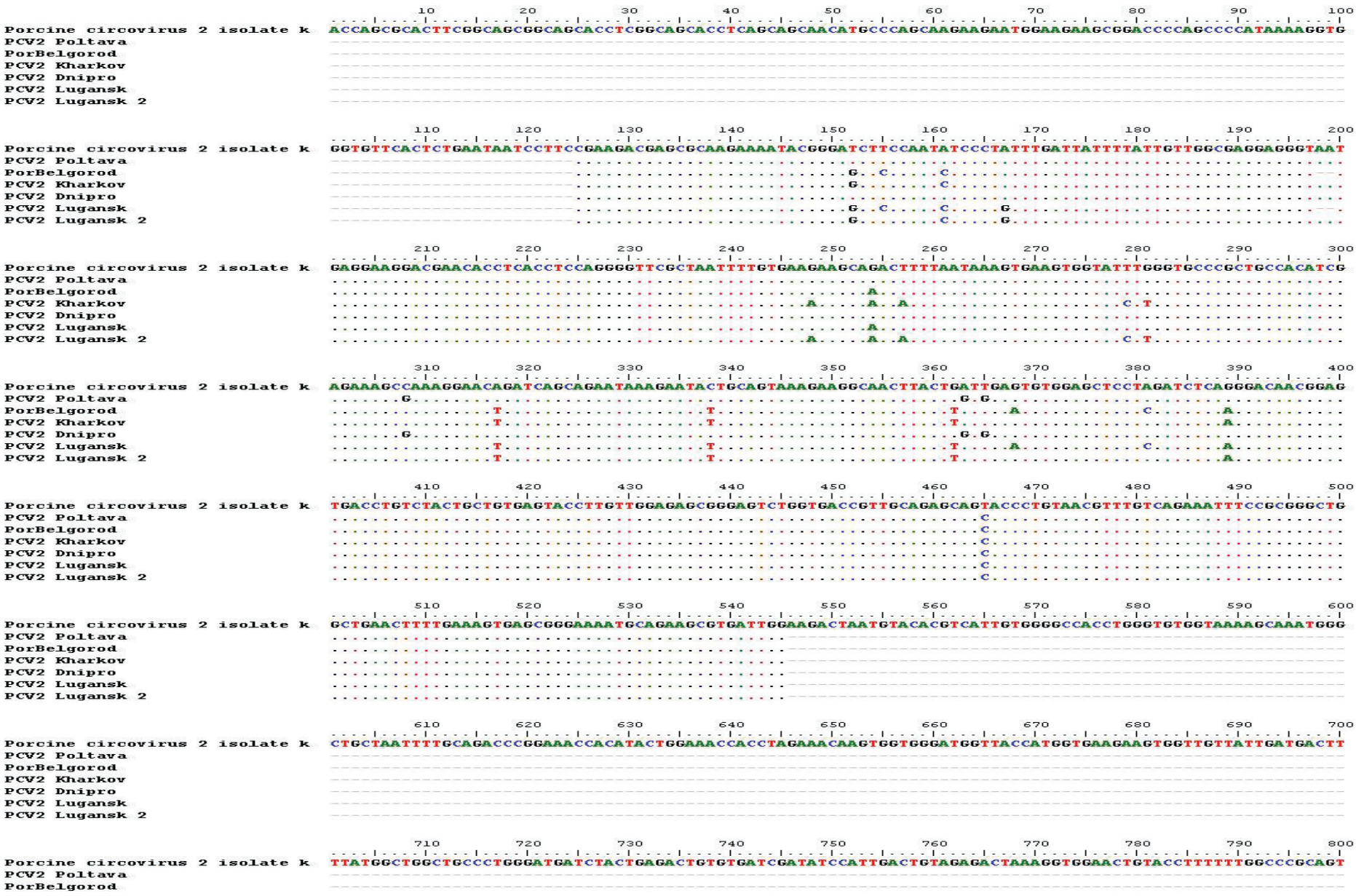

Figure 1. The multiple alignment of PCV-2 sequences derived from 421 bp *rep* gene fragment from Ukrainian isolates of the agent

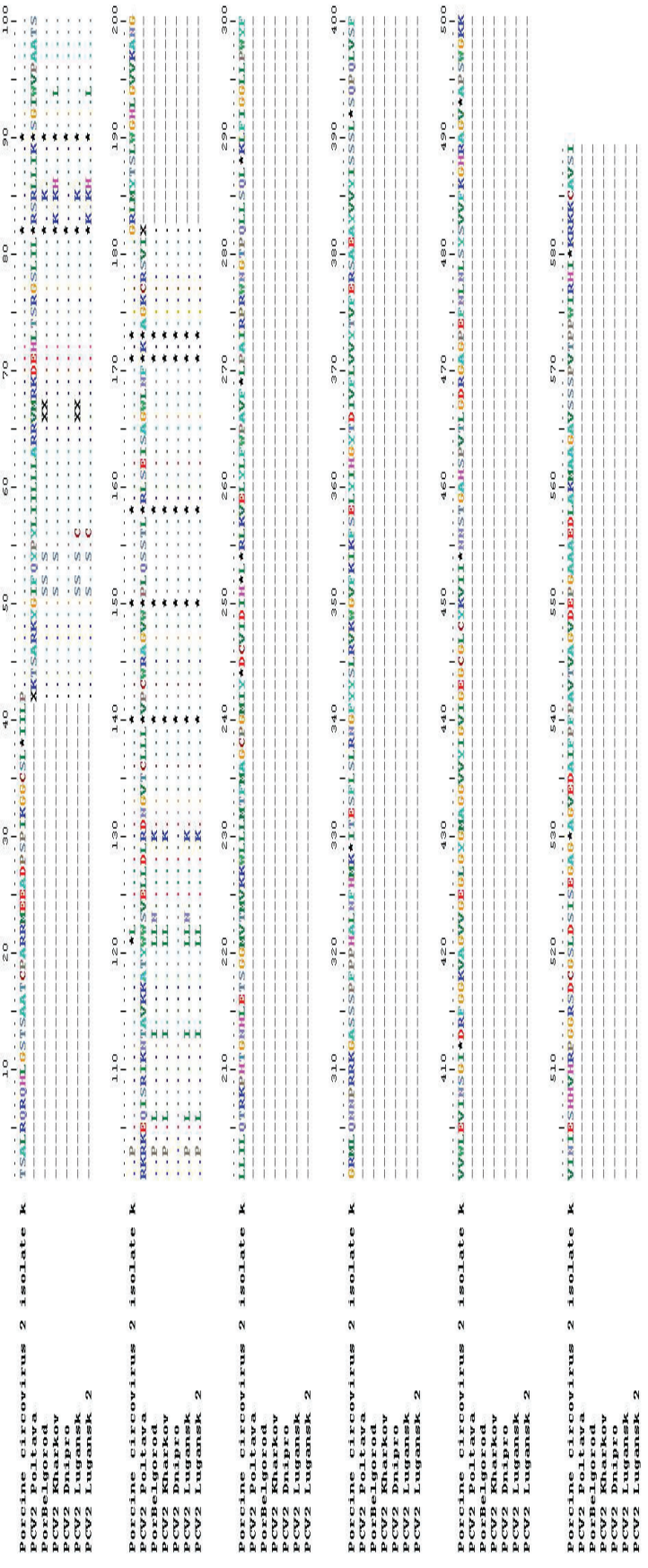

Figure 2. Amino acid sequence, derived from the sequenced 421 bp region of the replicative protein of the Ukrainian PCV-2 isolates

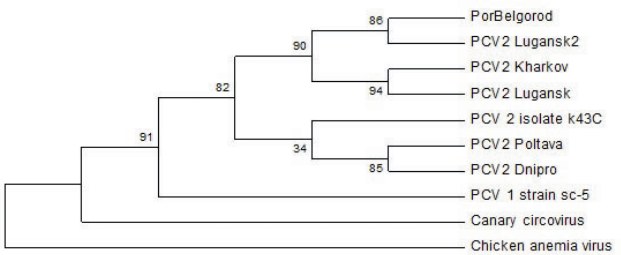

Figure 3. Rooted dendrogram of the Ukrainian PCV-2 isolates (*rep* gene)

The last step of our study included the creation of the phylogenetic trees from PCV-2 sequences of *rep* gene, allocated in the different locations using Neighbor Joining and Minimal evolution methods. The dendrograms were constructed using sequences, published already in the GeneBank. The out-group (canary circovirus and infectious chicken anemia virus, and PCV-1) sequences were used to create the rooted tree.

Pairwise Distances (G:\Porcine circovirus\PCV2 UA all seq.meg)

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(A,B)

	1	2	3	4	5	6	7
1. PCV 2 Dnipro							
2. PorBelgorod	0.032						
3. PCV 2 Kharkov	0.034	0.017					
4. PCV 2 Poltava	0.000	0.032	0.034				
5. PCV 2 Lugansk2	0.037	0.019	0.002	0.037			
6. PCV 2 Lugansk	0.034	0.002	0.019	0.034	0.017		
7. PCV 2 isolate k43C	0.010	0.027	0.029	0.010	0.032	0.029	

(PCV 2 Dnipro-PCV 2 Dnipro) / Nucleotide: Kimura 2-parameter

Figure 4. The distances between PCV-2 Ukrainian isolates (*rep* gene)

The created tree (Fig. 6) demonstrated belonging of the 1st group of analyzed Ukrainian viruses to the lineage of the American strains contaminant of the cell lines of genotype 2 PCV-2 (88%).

The group contained the isolates PorBelgorod, PCV2 Lugansk 2, PCV2 Kharkov and PCV 2 Lugansk belonged to genotype 1 strains with European origin (similarity 70–93%).

Test of Neutral Evolution (G:\Porcine circovirus\PCV2 UA all ...

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(A,B)

	1	2	3	4	5	6	7
1. PCV 2 Dnipro		0.309	1.234	0.000	1.078	0.479	0.336
2. PorBelgorod	0.758		1.256	0.309	1.044	1.081	0.622
3. PCV 2 Kharkov	0.220	0.212		1.234	1.080	1.044	1.876
4. PCV 2 Poltava	0.000	0.758	0.220		1.078	0.479	0.336
5. PCV 2 Lugansk2	0.283	0.299	0.282	0.283		1.261	1.721
6. PCV 2 Lugansk	0.633	0.282	0.299	0.633	0.210		0.444
7. PCV 2 isolate k43C	0.738	0.535	0.063	0.738	0.088	0.658	

(PCV 2 Dnipro-PCV 2 Dnipro) / Codon: Nei-Gojobori method (p-distance)

Figure 5. Neutrality evolution indexes in PCV-2 rep gene sequences comparison

The Minimal evolution method derived tree (Bootstrap 500) demonstrated lower likelihood concerning belonging of the 1st group isolates (only 25 and 54 % after condensation) (Fig. 7).

This could be explained by the big amount of genetically similar isolates that have different mismatches in the nucleotide sequences of the rep gene.

Isolates PorBelgorod and PCV2 Lugansk were situated also in the clade of the European viruses, and the common progenitor of them has been detected (am8 isolate). The Lugansk 2 isolate (82%) cold be potentially changed virus with the same origin and PCV2 Kharkov isolate.

The strains from the American genotype were mostly genetically related to am2 and am9 strains.

Conclusion. The study of the phylogenetic relations of PCV-2 Ukrainian populations based on the analysis of 421 bp region sequencing of the rep gene demonstrated their belonging to the lineage of the American origin cell cultures contaminated strains (88%) of the genotype 2. The group of sequences, contained DNA-amplicones of PCV-2 isolates PorBelgorod, PCV2 Lugansk 2, PCV2 Kharkov and PCV 2 Lugansk belonged to the lineage of European origin strains of genotype 1 (70–93%).

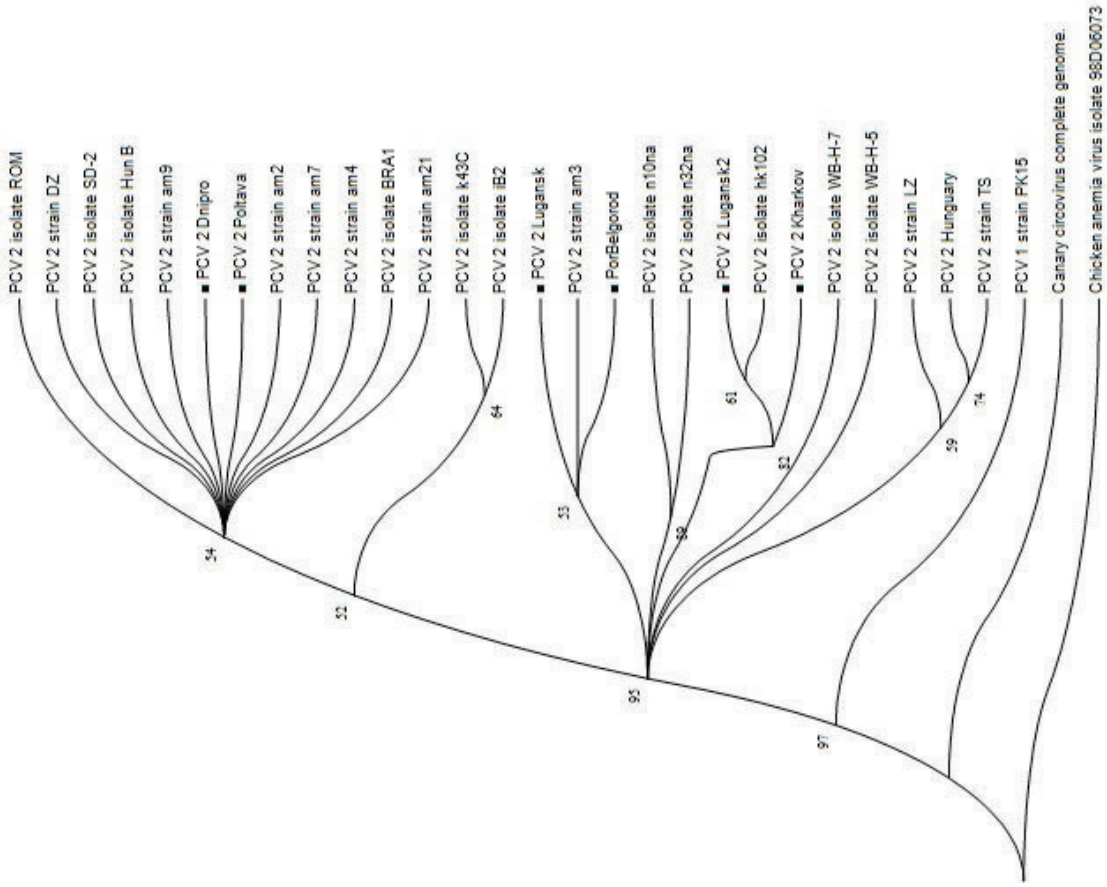

Figure 7. Condensed dendrogram of the phylogenetic relations of PCV-2 isolates and strains (Minimal evolution, ■ — Ukrainian isolates).

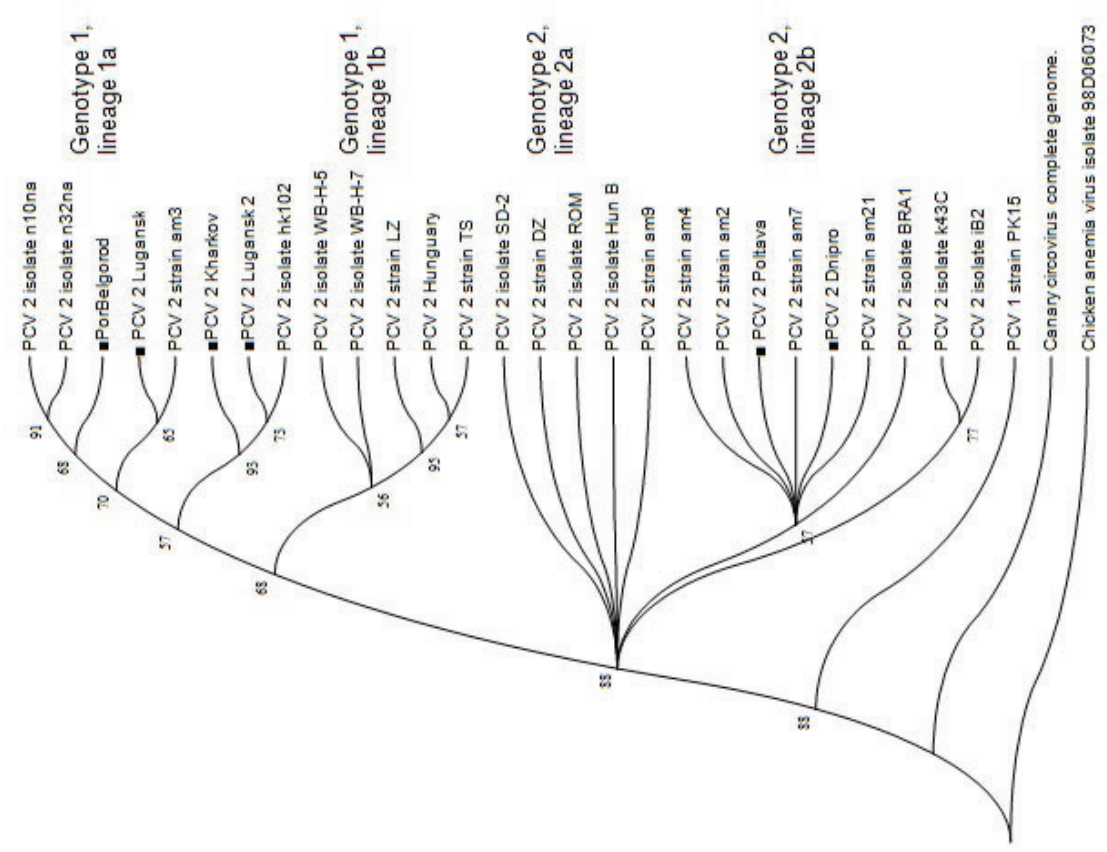

Figure 6. Dendrogram of the phylogenetic relations among the strains and isolates of PCV-2 (Neighbor Joining, ■ — Ukrainian isolates)

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