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PHYLOGENETIC AND MOLECULAR GENETIC STUDIES OF THE ANIMALS ARTERIVIRUSES

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Summary. This study was aimed to provide the phylogenetic characteristics of arteriviruses of the different species, and to perform PCR-based surveillance of the porcine diseases, caused by PRRS virus and its associates.

The study was conducted based on the molecular genetics methods, including PCR, phylogenetic analysis of sequences of the different arteriviral species.

The phylogenetic relationships of the porcine reproductive and respiratory syndrome virus circulating in different geographical regions were studied. The availability of the phylogenetic analysis for genotyping was performed and microorganism molecular markers were demonstrated. The monitoring of the pathogen PRRS spread in the farms of Eastern Ukraine was conducted.

Variability of genes encoding glycoproteins GP2, GP3, GP4 and GP5 of the arteriviruses of animals was demonstrated and allowed to recommend these for PRRSV molecular epidemiology study. The monitoring spread of the PRRS virus in the farms in the Eastern Ukraine demonstrated the possibility of PRRS virus association with porcine circovirus type 2.

Keywords: porcine reproductive and respiratory syndrome virus, real-time polymerase chain reaction, phylogenetic analysis.

Introduction. The most common event in the molecular evolution of biomacromolecules (DNA and RNA) are nucleotide substitutions, that accumulated by the independent evolution of sequences from a common ancestral form. The average number of the nucleotide substitutions for two homologous sequences of two type biological molecules of the organisms to the one nucleotide site determines the evolutionary distance. Setting the evolutionary distance is to find the differences of the genetic material and its relationship at the evolution. In the future, this knowledge makes it possible to build the phylogenetic trees, to determine the time of taxon divergence based on the comparison of the primary structures of the genetic biomacromolecules, to reconstruct the history of the biota, to study the recent changes, evolution of genes in the space and the time (Riddle, 1996; Hewitt, 2001). If the replacing is rare (or the evolution time is small), we can assume that the number of substitutions is directly proportional to the time of their evolution in a pair of sequences.

The topology of the constructed phylogenetic trees, based on the evolutionary distances, allows obtaining the reliable information, particularly, about the genotypic characteristic of the pathogen, spectrum of the infectious agent isolates circulating in a particular area.

These processes complicate greatly the genotyping of infectious agents and, therefore, require the careful study by the experts of veterinary medicine and molecular biology, this is important for the development of the livestock industry of the Ukraine, creation of the modern means for veterinary support of pig farming.

One of the most common and developed industries is the swine livestock today. A lot of swine viral diseases causing a negative impact to the animal reproduction system, including porcine reproductive and respiratory syndrome (PRRS) and porcine circovirus desease, caused by porcine circovirus type II (PCV-2) were described in Ukraine (Gerilovich et al., 2011). Diseases caused by these pathogens as monoinfection and in the associations, cause significant economic losses to pig production. In addition, the PRRS agent has hypervariable organization of genetic material that causes the interest of genotyping and studying genetic markers of the virus origin and pathogenicity.

The disease caused by a PRRS virus was discovered in 1967 in North Carolina (USA) and Canada, and a few years later – in Europe (1990 – in Germany, 1991 – in the UK) (Wensvoort et al., 1991). Today this contagious viral infection is widespread in a lot of countries in Southeast Asia, Europe and America. PRRS virus was first isolated in primary cultures of pig alveolar macrophages (PAMs) in 1991 (the Lelystad strain) (Lurchachaiwong et al., 2008).

There are two PRRS genotypes (each has its subtypes) – European (type I) and American (North American) (type II). Their genomic RNA was characterized by only (55-70) % similarity and may have different ways of the evolutionary development (Stadejek et al., 2006; Nelson et al., 1993. It is believed (Martínez-Lobo et al., 2011) that pathogens of both genotypes differ on some biological properties, including pathogenicity.

In particular, showed that type II isolates causing more severe respiratory disease than type I isolates (Martínez-Lobo et al., 2011). But numerous studies concerning the comparison of both genotypes isolates never carried out. These genotypes have permanent structural difference related concerning the amino acids ORF7 (open reading frame) at positions 123 and 127, and the differences in replication concerning ORF1a and 5-noncoding region of genomic RNA. In addition, the genotypes of the PRRS virus differ in serological cross-reactions (Murtaugh et al., 1998).

Other arterivirus – arteritis virus – often found in populations of horses in different countries, but information concerning the genetic variability of this virus is almost absent. Only in 1999 was suggested the possible existence of separate geographical groups of the virus by studying of 22 strains of EAV from North America and Europe (Stadejek et al., 1999).

The aim of this work is to study the phylogenetic relationships of the animal arteriviruses and the monitoring of the PRRS and PCV-II viruses spread in the swine livestock in the eastern region of Ukraine by real-time PCR.

Materials and Methods. Mega 4, ver. 4.0.2 (Tamura et al., 2007); POWER, ver. 1.0 (PhylOgenetic WEb Repeater (POWER), 2005); PhyML ver. 3.0 program were used for the phylogenetic analysis (Dereeper et al., 2008). To build a traditional dendrograms based on gene sequences and genomic RNA paramyxoviruses used remote-matrix method – a method of binding neighbour joining and maximum parsimony. To test the reliability of the received dendrogram topology used bootstrap.

We have selected fully and partially sequenced genomic RNA sequence and basic arteriviruses gene of the genus Arterivirus for the phylogeographic studies of the arteriviruses from the farm animals: 1) porcine reproductive and respiratory syndrome virus (PRRSV); 2) equine arteritis virus (EAV). All sequences obtained in the FASTA (*.fasta) or GenBank (*.gb), that allowed to use modern bio-molecular software (including on-line) for pair and multiple, local and global alignment to determine conserved and variable fragments of genes and insertions, mutations and deletions, to build the dendrograms and to appropriate phylogenetic analysis (Abramson, 2007; Lukashov, 2009). Clinical material from pigs of different gender and age groups of livestock has been collected in the eastern region of Ukraine and pathological material from dead animals or aborted fetus during 2013-2014. Viral RNA and DNA extraction was performed by affinity sorption. Reverse transcription reaction was performed by using «First Strand cDNA Synthesis Kit» (Thermo SCIENTIFIC, USA).

For the elaboration of specific DNA and cDNA was used real-time PCR with the commercial kit «Maxima SYBR Green / ROX qPCR» (Thermo SCIENTIFIC, USA). For setting reaction was used the primers system (Kleiboeker, 2004). Amplification was performed on Thermocyclers DT lite («DNA technology», Russian Federation) at the next time and temperature parameters:

For PCV-2
1 step: denaturation – 94 °C – 2 min– 1 cycle;
2 step: denaturation – 94 °C – 15 sec;
annealing – 58 °C – 1 min,
synthesis – 72 °C – 20 sec,
detection – 72 °C – 12 sec;
} 40 cycles

For PRRS virus:
1 step: denaturation – 94 °C – 2 min – 1 cycle;
2 step: denaturation – 94 °C – 15 sec;
annealing – 60 °C – 1 min,
synthesis – 72 °C – 20 sec,
detection – 72 °C – 12 sec.
} 40 cycles

Results and Discussion. Arteriviral genome presented by single-helix non-segmented RNA molecule with positive polarity, length is about 15 thousand nucleotides, that encodes the virus structural proteins, including four glycoproteins (GP2, GP3, GP4 and GP5), two non-glycosylated structural nvelope proteins (E and M), non-structural proteins Nsps, which are crucial for viral replication and immune modulation, and nucleocapsid protein N (Chen et al., 2011; Li et al., 2011). Multiple aligned arteriviruses genomic RNA sequences circulating in different geographical regions and represented in the international databases, demonstrated the least conservative genes are genes that encode proteins GP3 and GP5. This is the basis for using sequences of these genes for genotyping based on the results of the phylogenetic analysis.

The most conserved were genes that encode the proteins M and N, this agrees with the data of works (Snijder and Meulenberg, 1998; Grebennikova et al., 2004). Phylogenetic analysis based on genes sequences encoding protein M, demonstrated high level of similarity for different arteriviruses (Fig. 1, cluster 1), that was the porcine respiratory reproductive syndrome virus, which circulates in the Canada (ES- 437 022 isolate) and in the China (HN-09 strain), and equine arteritis virus, which circulates in the United States (S4216 isolate) and in the France (strain F62).

The topology analysis of the phylogenetic tree based on the sequences of genes encoding N protein, showed the impossibility of the porcine respiratory reproductive syndrome two main virus genotypes - North American and European differentiation. Because, the representatives of the different pathogen genotypes belonging to the same cluster, and branches that correspond to isolate of the North American PRRS virus genotype, localized within the cluster (Fig. 2).

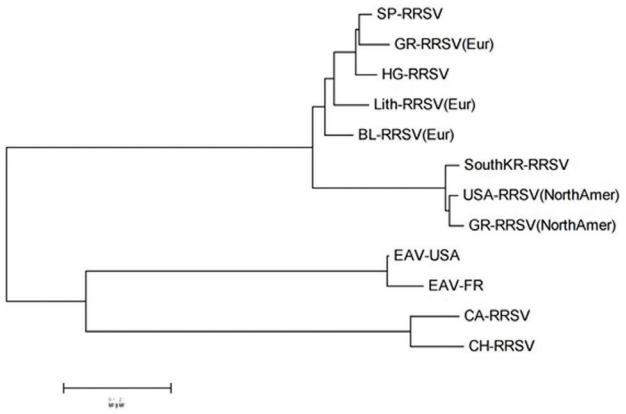

Figure 1 – Phylogenetic tree based on genes sequences encoding a M protein of the arteriviruses, which circulates in the different countries (USA – United States; BL – Belarus; CA – Canada; CH – China; SP – Spain; GR – Germany; HG – Hungary; Lith – Lithuania; SouthKR – South Korea; FR – France; Eur – European genotype; NorthAmer – NorthAmerican genotype)

Figure 2 – Phylogenetic tree based on genes sequences encoding a protein N of the arteriviruses, which circulates in the different countries (USA – United States; BL – Belarus; CA – Canada; CH – China; GR – Germany; HG – Hungary; Lith – Lithuania; SouthKR – South Korea; FR – France; IT – Italy; ROM – Romania; SLV – Slovakia; GB – United Kingdom; SL – Slovenia; SB – Serbia; AU – Austria; RU – Russian Federation; CZ – Czech Republic; TL – Thailand; PL – Poland; SP – Spain; BU – Bhutan; HR – Croatia; VN – Vietnam; TR – Turkey; Eur – European genotype; NorthAmer – NorthAmerican genotype)

This topology of the phylogenetic tree indicates a high level of similarity of the genes sequences encoding protein N, and therefore a large number of conservative structures in this protein that consistent with the results of work (Grebennikova et al., 2004b).

To study the phylogenetic relationships of the arteriviruses we have selected 16 fully sequenced PRRS virus genomic RNA sequences, 3 – equine arteritis virus circulating in a different geographical regions and sequences of genes encoding glycoproteins GP2, GP3, GP4 and GP5 of the arteriviruses, which role in joining the virus to permissive cells, causing a viral pathogenesis, an apoptosis, increased antibody depends is not been fully elucidated. The linear topology of the phylogenetic tree showed on the Fig. 3 indicates the origin of known PRRS virus genotypes from a common ancestor, that is consistent with the results of work (Grebennikova et al., 2004a), whose authors studied the primary structure of the pathogens genome of the both genotypes circulating in Poland and Lithuania.

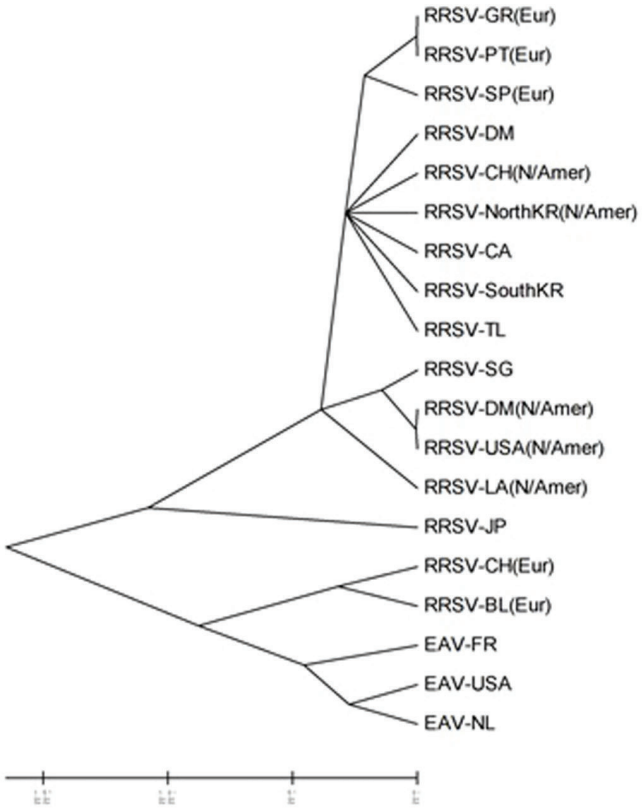

Figure 3 – Phylogenetic tree based on fully sequenced genome RNA sequences of the arteriviruses, which circulates in the different countries (USA – United States; BL – Belarus; CH – China; GR – Germany; South KR – South Korea; FR – France; TL – Thailand; SP – Spain; PT – Portugal; DM – Denmark; North KR – North Korea; CA – Canada; SG – Singapore; LA – Laos; JP – Japan; NL – Netherlands; Eur – European genotype; N / Amer – North American genotype)

The presence of two clusters for members of the European PRRS virus genotype and two clusters (Fig. 3) for members of the American pathogen agent genotype indicates the possibility of the existence at least two subtypes for each genotype. PRRS virus isolates that make up a particular subtype, have not only a high level of similarity of the genomic RNA primary structure, but perhaps have the common immunobiological properties that were the object of study in recent years (Kukushkin et al., 2004).

According to the conducted phylogenetic analysis of the arteriviruses we can conclude the phylogenetic proximity of the equine arteritis virus which isolates form separated cluster on the phylogenetic tree (Fig. 3) to one of the subtypes of the European genotype PRRS virus.

The most significant for the genotyping arteriviruses of the animals is a gene encoding a protein GP5. This glycoprotein is the major coat protein of the PRRS virus with molecular weight of 26 kDa, consisting of about 200 aminoacids residues. It is known (Kapur et al., 1996; Andreyev et al., 1997) that the GP5 protein is highly polymorphic, being under the constant pressure of selection due to its open position on the virions outer surface (Meulenberg et al., 1995). Due to its polymorphic nature the GP5 protein is considered as the main molecule in creating subunit vaccines. The aminoacid sequences of the ORF5 open reading frame have two hypervariable regions, one of which is localized in the signal peptide. Thanks GP5 polymorphism, the gene encoding this protein is highly informative regarding the evolution and origin of different PRRS strains and considered as a target for the analysis of genetic diversity not only PRRS virus, but the equine arteritis virus.

The complex nature of the encoding the protein GP5 gene, allows to consider this gene as the main subject in the study evolutionary relationships of the arteriviruses. However, the results of the arteriviruses phylogenetic analysis (circulating in different geographical regions) based on sequences of genes encoding proteins GP2 (Fig. 4), GP3, GP4, that were established, and compare them with the results of the phylogenetic analysis based on the sequences of the gene encoding glycoprotein GP5, convincing the possibility of the arteriviruses differentiation and PRRS virus genotyping (type determination) based on these genes. It is important that each PRRS virus genotype, as the equine arteritis virus isolates, forms separated cluster on the dendrogram.

The early diagnosis of the swine viral diseases is a necessary condition for the effective development of a pig production as one of the promising sectors of Ukraine livestock. It is important to timely identification of patients and latently sick animals, in that it affects the effectiveness of a livestock treatment. One of the modern and fast methods for the detecting infectious agents in animals, even in the early stages of the disease, is the real-time polymerase chain reaction (RT-PCR).

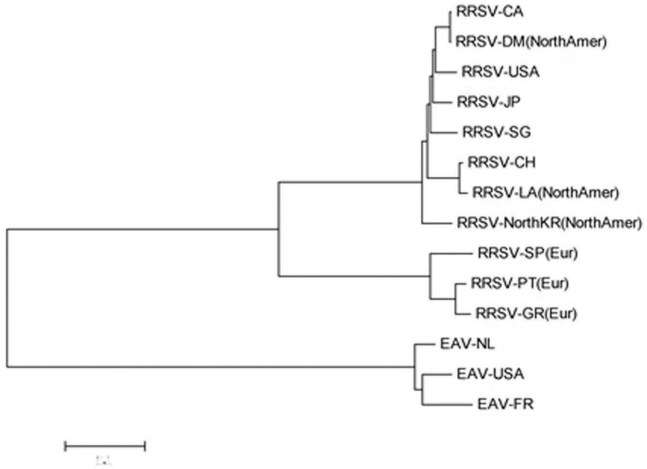

Figure 4 – Phylogenetic tree based on genes sequences encoding G2 protein of the arteriviruses, circulating in the different countries (USA – United States; CH – China; GR – Germany; PT – Portugal; DM – Denmark; North KR – North Korea; CA – Canada; SG – Singapore; LA – Laos; JP – Japan; NL – Netherlands; FR – France; SP – Spain; Eur – European genotype; N / Amer – North American genotype)

The results of our studies suggest the prevalence of respiratory reproductive disorders in pigs in the Ukraine caused by PRRS virus, PCV-2 or their associations (Gerilovich et al., 2011). Clinical signs of disease were extremely diverse in the study of epizootic state of farms, where more often met PRRS associations and PCV-2 associations, respiratory disorders appeared in the young animals, while adult cattle recorded the disorders of the reproductive system.

126 samples of various materials were tested by real-time PCR during 2013-2014. We observed the fluorescence signal for 65 samples only with primers that flank the specific PRRS virus fragment (Fig. 5); 23 samples contained only PCV-2 genetic material (Fig. 6).

We observed the fluorescence signal for 17 samples with primers system, which the target was the PRRS virus fragment, and with a primers system flanking specific fragment of the PCV-2. Therefore, these samples contained genetic material of both pathogens, and diseases of animals, which samples were selected, due to the association of these viruses.

number of wells	nomekr tube	Cp. Fam	Cp. Hex	results
A1	sample 1	17,3		+
A2	sample 2	20,5		+
A3	K+	16,8		+
A4	K-			-

Dependence of the FAM channel fluorescence from cycle number

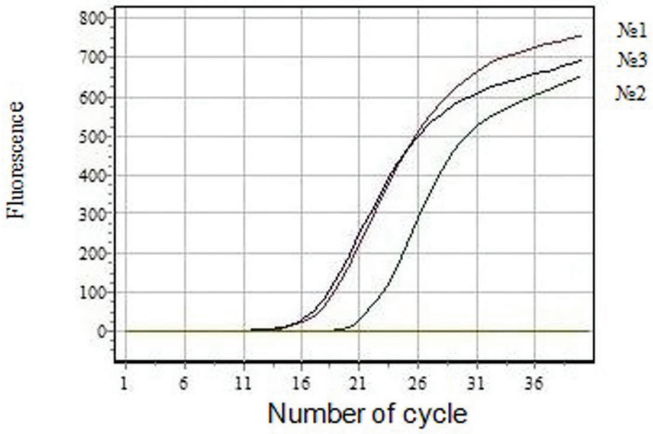

Figure 5 – Amplification curves of specific cDNA fragment of the PRRS virus with using the intercalating dye SYBR – Green. № 1, № 2 – amplification curves of specific cDNA fragment PRRS virus from samples taken from animals; № 3 – amplification curve of the positive control.

number of wells	nomekr tube	Cp. Fam	Cp. Hex	results
A1	sample 1	16,5		+
A2	sample 2			-
A3	K+	21,1		+
A4	K-			-

Dependence of the FAM channel fluorescence from cycle number

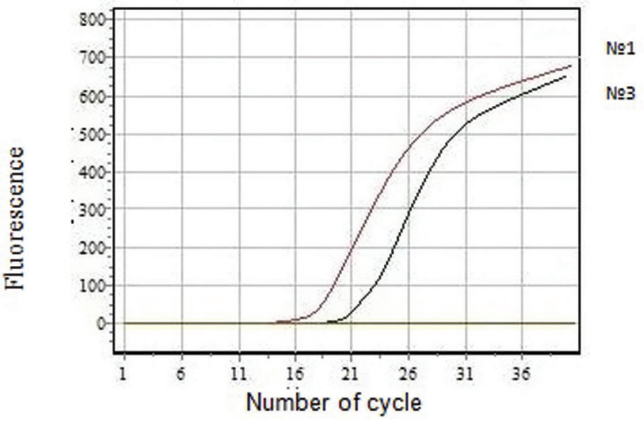

Figure 6 – Amplification curves of specific cDNA fragment of the PCV-2 virus with the intercalating dye SYBR – Green using. № 1, № 2 – amplification curves of specific cDNA fragment PRRS virus from samples taken from animals; № 3 – amplification curve of the positive control.

Thus, it was proved the variability of genes encoding glycoproteins GP2, GP3, GP4 and GP5 of the arteriviruses of animals. The phylogenetic analysis of the arteriviruses demonstrated the possibility of differentiation and, in particular, the PRRS virus genotyping based on these genes. The monitoring spread of the PRRS virus in the farms in the Eastern Ukraine was held, the possibility of PRRS virus association with porcine circovirus type 2 was shown.

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