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EXPERIMENTAL WORKS

Molecular Genetic Analysis of Genomes of Porcine Respiratory and Reproductive Syndrome Viruses and Porcine Circovirus Type 2 Circulating on the Territory of the Russian Federation

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Abstract—A molecular genetic analysis of genomes of porcine reproductive respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2) circulating in the territory of the Russian Federation is given. The results of the study showed the presence of circulation of European genotypes of PRRSV strains that are similar to isolates found in France and Denmark from 1998 to 2001. We explained the homology of a fragment of one of the genes between the Russian isolates and the vaccine strain used in Porcilis PRRS (Intervet) vaccine, but this requires further study. Strains of the North American genotype of PRRS were not found. The PCV-2 genomes fall into three separate groups. One (genotype 2b) is formed by isolates in Malaysia, Brazil, Switzerland, China, Slovakia, the United Kingdom, and the United States, which were isolated during the period from 2004 to the present time. The second group consists of the field sequences of viruses isolated in 2000–2012 in Canada, the United States, China, and South Korea (genotype 2a). The third group is formed by highly pathogenic isolates evolved in 2013 in China (genotype 2c). The circulation of all three known genotypes of PCV-2—2a, 2b, and 2c—in the Russian Federation was shown.

Keywords: molecular genetic analysis, phylogenetic analysis, virus of porcine respiratory reproductive syndrome, porcine circovirus type 2

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INTRODUCTION

Disturbances of the ability of pigs to reproduce are causing serious economic damage to pig farming enterprises all over the world. Respiratory illnesses are carried by 30–70% of piglets, and mortality can reach 40% [1]. The most widespread virus agents of reproductive pathologies of pigs are porcine reproductive respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2).

PRRS is a viral contagious disease characterized by disorder of respiratory and reproductive functions. The disease has been known since 1980 and was first registered in Canada. Currently, PRRS is a disease found on almost all continents where developed pig farming is present [2]. Recently, PRRS circulation has become panzootic. Only Australia and Oceania can be considered exceptions [3].

The disease pathogen is an RNA-containing enveloped virus with a genome size of 15.1–15.4 thousand nucleotides that belongs to the *Arterivirus* genus, Arteriviridae family, *Nidovirales* sequence. At present, two PRRS genotypes have been discovered—European and American, the prototypes of which are the Lelystad and VR-2332 strains, respectively. These two genotypes of PRRS virus have from 50 to 80% homology at the nucleotide level [4, 5]. The European and North American virus types seem to have had different evolutionary histories, as their genomes differ considerably [6]. The distinguishing virus feature is variability of the genomes of its isolates [7, 8]. Currently, on the European and American continents, there are two virus genotypes simultaneously. In addition, a highly virulent PRRS virus has emerged in China [9].

PCV is a viral disease mainly of piglets. PCV-2 is found among pigs with different diseases, including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine reproductive disorders and respiratory illnesses. Retrospective serological studies have shown that PCV-2 has been circulating in pig farms since 1969 [10].

The PCV-2 pathogen belongs to the *Circovirus* genus, Circoviridae family. It is a tiny nonenveloped icosahedral virus containing closed unispiral genome DNA of 1767–1768 nucleotides in size. In addition to pathogenic PCV-2, there is nonpathogenic PCV-1, which was discovered as a RK-15 noncytopathogenic

contaminant of interweaving culture of piglets' kidney cells [11]. The homology of their genomes is 76% [12]. On the basis of genetic differences in the second open reading frame (ORF2), PCV-2 is divided into the genotypes PCV-2a, PCV-2b, and PCV-2c. Currently, differences in disease severity are connected with the change of genotype from PCV-2a to PCV-b and PCV-2c [13].

Today, regular studies on circulation of PRRSV and PCV-2 are not carried out, and the data on the character of variations of virus genomes are not complete. The aim of our study was to conduct a phylogenetic analysis of fragments of the genomes of PRSS and PCV-2 circulating in different regions of the Russian Federation.

MATERIALS AND METHODS

Material for study. During the work, we studied 170 samples of blood serum of piglets at an age of 100–110 days. The samples were obtained in eight different regions of the Russian Federation. Before the research, the samples were kept without additional reagents at –70°C. To clean the obtained samples of remains of erythrocytes, they were centrifuged.

Isolation of nucleic acids of PRRS and PCV-2 was carried out with the use of Trizol Reagent (Life Technology, United States) and a commercial kit for DNA isolation (Vetbiokhim, Russia) with the use of nonorganic sorbent in accordance with the methodology of the manufacturer [14].

Detection of genome of PRRSV in the studying samples was conducted with the use of the Test System for Detection of PRRS by PCR (Vetbiokhim, Russia) [14]. The reaction mixture contained 5 mL of RNA 10 pmol of each primer, 0.25 mM of each dNTP, 2.5 units of Taq-polymerase, 50 units of MMLV-reverse transcriptase, 40 units of inhibitors of ribonuclease, 10 mM of tris-Hcl (pH 9.0), 50 mM of KCl, 0.1% triton X-100, and 1.5 mM of $MgCl_2$. To carry out RT-PCR, we used the following temperature regimes: 50°C, 45 min and 94°C, 5 min and 25 cycles: 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s. After RT-PCR, we conducted a second round of amplification. The reaction mixture contained 5 mL of RT-PCR product, 10 pmol of each primer for PCR-II, 0.25 mM of each dNTR, 2.5 units of Taq-polymerase, 10 mM of tris-HCl (pH 9.0), 50 mM of KCl, 0.1% triton X-100, and 1.5 mM of $MgCl_2$. We used the following temperature parameters of PCR: 25 cycles: 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s.

Detection of the PCV-2 genome was conducted with the use of the Test System for Detection of PCV-2 by PCR (Vetbiokhim, Russia). The reaction mixture for PCR contained 0.25 mM of each dNTP, 2.5 units of Taq-polymerase, 10 mM of tris-HCl (pH 9.0), 50 mM of KCl, 0.1% triton X-100, and 1.5 mM of $MgCl_2$. We used the following temperature regimes: 35 circles: 94°C, 45 s; 55°C, 45 s; and 72°C, 45 s and 1 circle: 94°C, 45 s; 55°C, 45 s; and 72°C, 5 min.

Results of investigating blood serum samples of piglets at the age of 100–110 days for the presence of genomes of PRRSV and PCV-2 by the PCR method

Region	Number of studied samples	Number/% of samples positive for the presence of PRRSV	Number/% of samples positive for the presence of PCV-2
Moscow oblast	20	13/65	16/80
Tomsk oblast	10	3/30	6/60
Tyumen oblast	15	4/26.7	5/33.3
Sverdlovsk oblast	60	17/28.7	19/31.7
Novgorod oblast	15	5/33.3	8/53.3
Yaroslavl oblast	10	2/20	2/20
Samara oblast	25	8/32	7/28
Perm oblast	15	7/46.6	9/60
Total	170	62/35.5	72/42.3

PCR and RT-PCR were done on a Tertsik amplifier (DNA-technology, Russia).

Analysis of PCR fragments was conducted by the electrophoresis method in 2% agarose gel containing ethidium bromide in a concentration of 0.4–0.5 μg/mL. The gels were photographed and analyzed using a transilluminator with ultraviolet light with waves 254 nm long. The size of the fragment of the PRRSV genome was 403 pn, while the fragment of the PCV-2 genome was 356 pn. PCR products were isolated from the gel with the GeneClean Kit for cleaning PCR products (Bio-101 Inc., United States) in accordance with the manufacturer's instructions, and the nucleotide sequence was described. The primers used for sequencing were the same as for PCR-2—PRRSV and PCV-2, respectively.

Determination of nucleotide sequences was carried out on an ABIPRISM 3130 automatic sequencer (Applied Biosystems, United States) following the manufacturer's recommendation.

In the construction of phylogenetic dendrograms, the nucleotide sequences ORF-7 of PRRSV and ORF-2 of PCV-2 represented in the NCBI GenBank database were equalized by the SeqMan program (Lasergene, United States). To construct the phylogenetic trees, we used the DNASTAR v.3.12 software package (Lasergene, United States).

RESULTS AND DISCUSSIONS

We studied samples of blood serum of piglets of different ages from different farms of the Russian Feder-

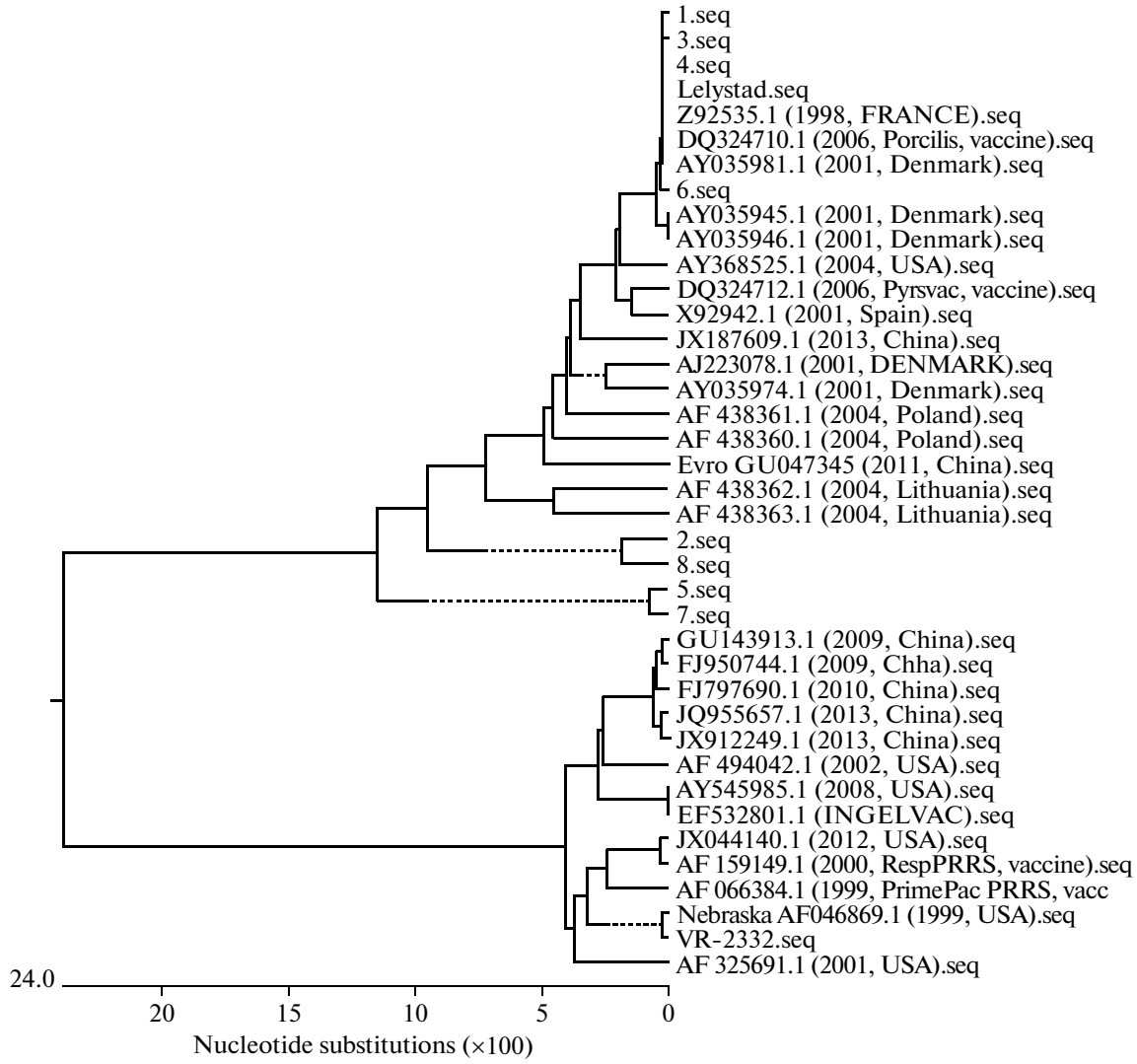

Fig. 1. Phylogenetic dendrogram obtained on the basis of comparative analysis of ORF-7 genome fragments of PRRSV (376 pn).

ation. The table shows that both PRRSV and PCV-2 were detected in all eight studied regions. The amount of positive results in the detection of PRRSV varied from 20% (Yaroslavl oblast) to 80% (Moscow oblast), being 42.3% on average (table).

To detect genetic differences between genomes of viruses of porcine respiratory diseases, we then carried out analysis of the nucleotide sequences of the ORF-7 fragment of PRRSV and ORF-2 of PCV-2, respectively. We obtained the nucleotide sequences of PRRSV and PCV-2 described above from the GenBank database. The length of the sequenced PCR fragments was 376 pn for PRRS and 307 pn for PCV-2.

Figure 1 shows a phylogenetic dendrogram designed on the basis of the analysis of results of sequencing of gene NP (ORF-7) fragments of PRRS virus. Dendrogram analysis showed that genomes of viruses 1, 3, 4, and 6 (Moscow, Tyumen, Sverdlovsk, and Yaroslavl oblasts) form a unified genetic group of isolates of European type of PRRS together with well-known sequences (Z92535, DQ324710, AY035981, M96262). This group includes field isolates of PRRS virus found on the territories of France and Denmark from 1998 to 2001. The homology of these isolates with the vaccine strain used in the vaccine Porcilis PRRS (Intervet) demands further study. It is known that the effectiveness of using live vaccines is complicated by the high variability of PRRS virus and possible reversion to wild virus type [8, 15]. Thus, in 1996 in Denmark, the use of live RespPRRS/Repro vaccine caused infection of 40% of pigs as attenuated virus reverted to wild type [16].

Viruses 2, 5, 7, and 8 (Tomsk, Novgorod, Samara, and Perm oblasts) make up a separate genetic group. They differ from each other and from other isolated sequences, for which reason we can say that there is a separate group of isolates of PRRS virus of European genotype typical for the Russian Federation. It is

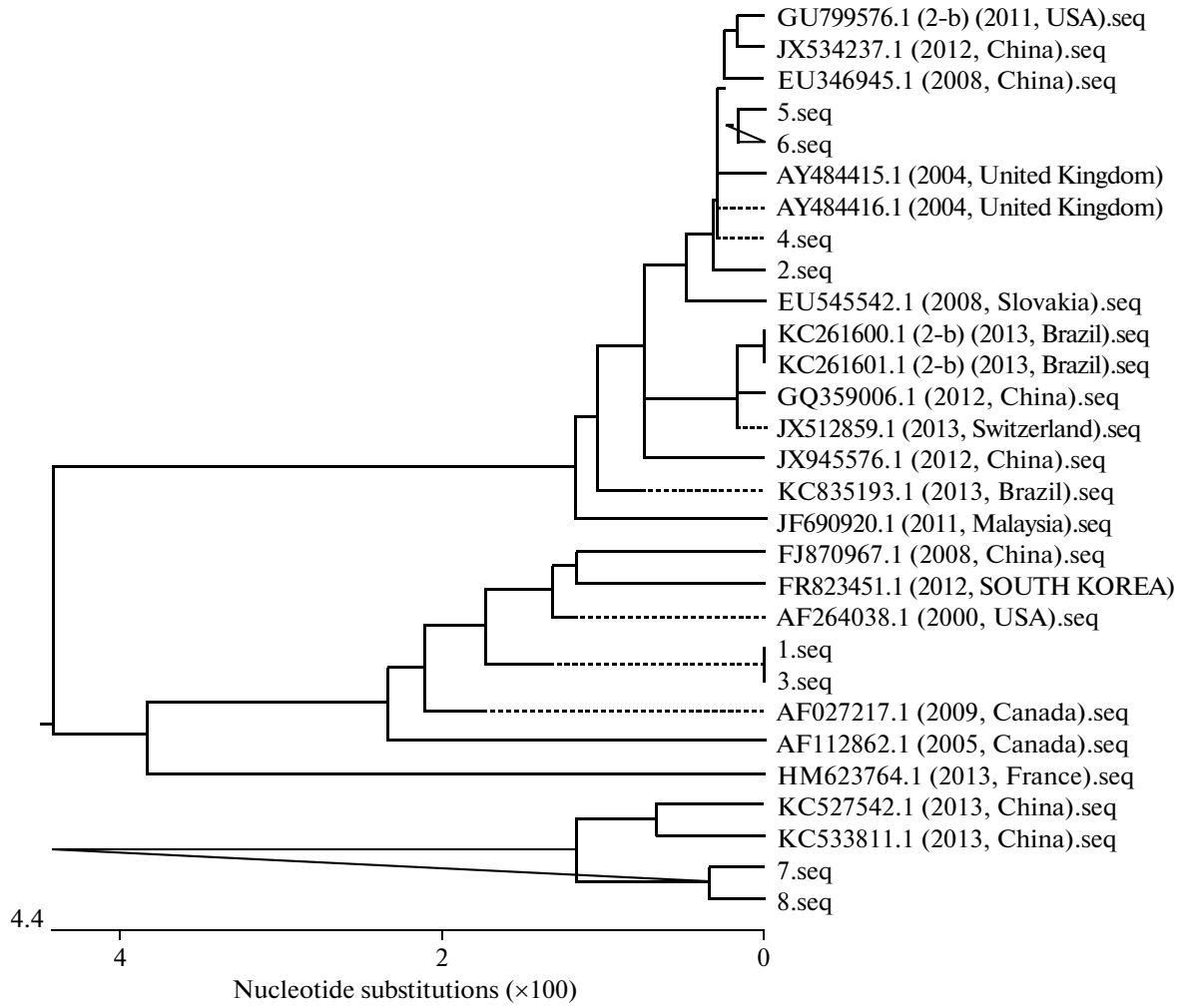

Fig. 2. Phylogenetic dendrogram obtained on the basis of comparative analysis of ORF-2 genome fragments of PCV-2 (307 pn).

interesting that we discovered that isolates of European genotype found in Spain were circulating in the territory of Russia and Belarus in 2001–2006 [17]. Studies of Russian genotypes of PRRS virus testify to the high variability of the virus.

In conducting the research, we did not find North American genotypes of PRRS virus. However, this does not mean that they are not present on the territory of the Russian Federation. They likely circulate in regions that we did not study.

Figure 2 shows a phylogenetic dendrogram designed on the basis of a sequence of the ORF-2 gene fragment of PCV-2. We analyzed it and discovered that the sequences of genome fragments of viruses 2, 4, 5, and 6 (from Tomsk, Sverdlovsk, Novgorod, and Yaroslavl oblasts) are very close to earlier known sequences (AY484415, JX534237, EU346945, GU799576, AY484416, EU545542, KC261600, KC261601, CQ359006, JX512859, JX945576, KC835193, and JF690920) and form one genetic group. This group includes viral isolates of PCV-2 found in Malaysia, Switzerland, China, Slovakia, the United Kingdom, and the United States during the period from 2004 to the present time. They belong to the genotype of PVC-2b.

The sequences of viruses 1 and 3 (Moscow and Tyumen oblasts) are similar to the sequences from GenBank FJ870967, FR82345, AF264038, AF027217, and AF112862 and belong to another genetic group that includes viruses from the genotype PCV-2a that were isolated in Canada, the United States, China, and South Korea in 2000–2012. We have earlier described the circulation of genotypes 2a and 2b on the territory of the Russian Federation [18]. The sequences of isolates 7 and 8 (Samara and Perm oblasts) are close to the sequences of isolates (KC527542 and KC533811) detected in China in 2013. We should add they these isolates belong to the new highly pathogenic PCV-2c strain. According to the study results, we can affirm the circulation of three well-known genotypes of

PCV—2a, 2b, and 2c—on the territory of the Russian Federation.

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