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PHYLOGENETIC ANALYSIS OF RUSSIAN EIAV ISOLATES USING AN OPTIMIZED NESTED PCR

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Abstract

Equine infectious anemia (EIA) is a viral disease wich affects all members of Equine species and is caused by a virus belonging to the genus *Lentivirus* in the family *Retroviridae*. This disease has a worldwide distribution, and its epizooty is registered in all continents. AGID positively reacting animals are revealed at planned serological studies in the Russian Federation from year to year. At the moment, a lot of attention is paid to control of the EIA virus prevalence, therefore, all over the world there are numerous studies to identify the virus and determine its molecular and genetic characteristics and origin. This paper presents data on the development of test systems based on PCR, which allows not only to identify the EIA viral genome in various samples of biological material, but also to carry out further sequencing the genome fragments of different EIA strains and isolates. EIA virus genome consists of three major genes, *gag*, *pol*, *env* (5′→3′). And the most useful marker for studying genetic diversity between different EIAV strains is a *gag*-gene necessary for intracellular virus assembly and release from cells. For carrying out the molecular genetic studies, we optimized nested PCR without reverse transcription and designed specific oligonucleotide primers for the second round of the reaction which flanking 1018 bp of proviral DNA of *gag*-gene. In the research we also determined the nucleotide sequence and phylogenetic relationships between previously isolated Russian EIAV strain and two modern isolates revealed in Russia. The findings allowed assigning 3-K-VNIITIBP-VIEV EIAV strain to group of North American origin with homology of 98 %. Isolates Nizhniy Novgorod-2011 and Omsk-2012 were classified as isolates of European origin with 82-83 % homology. Thus, application of PCR and a phylogenetic analysis allows to establish the belonging of new virus isolates to a certain genogroups and to assume their geographical origin. This information is necessary to determine the source and ways of distribution of the pathogen for the purpose to predict future epidemic situation and planning measures against the disease.

Keywords: EIAV, PCR, phylogenetic analysis.

Equine infectious anemia (EIA) is a disease of solipeds that is associated with the failure of hematopoietic organs and is manifested with recurrent or persistent fever, anemia and impaired cardiovascular function [1-4]. The EIA etiological agent is of viral nature (Equine infectious anemia virus — EIAV) and belongs to the genus *Lentivirus* in the family *Retroviridae* [4, 5]. The EIA viral genome consists of two positive-sense identical single-stranded linear RNA molecules [4, 6-8] and includes three major structural genes arranged (in 5′→3′) as *gag* (encode virion core proteins required for intracellular assembly and virus release from the cells), *pol* (encodes polypeptides with revertase, specific RNA-ase and DNA-endonuclease activity) and *env* (encodes surface virion glycoproteins involved in the formation of the virion envelope, in specific recognition of host cell and in virus penetration through its membrane) [4, 8, 9]. Furthermore, the EIA viral genome contains three additional open reading frames, the *tat*, *rev*, and *S2*, from which small accessory proteins involved in the viral RNA synthesis regulation and processing are expressed. The auxiliary protein S2 is an important determinant of the EIA virus replication and pathogenic properties in vivo [4, 6, 8-10]. The nucleotide sequence of the viral genome has long terminal repeats

(LTR) at both ends that are necessary for the integration of proviral DNA (pDNA) into the cell genome [8, 11].

The members of the *Equidae* family (horses, ponies, donkeys, mules and hinnies) are susceptible to the EIA virus [1, 2, 12-15]. Infectious anemia adversely affects industrial horse breeding. Among the 11 equine infections, EIA is included in the Organization for Animal Health list (Office International des Epizooties, Paris, OIE) which consists of the diseases the outbreaks of which must be reported by countries to OIE [8, 16].

Due to the registration of the disease at almost all continents [17, 18-20], much attention is paid to the problem of the EIA virus prevalence control. Phylogenetic analysis of local isolates and strains performed in different countries suggests that there are genetic subtypes of the EIA virus that circulate in the world currently [8, 14, 17].

By a diffusion precipitation (agar-gel immunodiffusion, AGID test), the positively reacting animals are revealed at planned serological studies in the Russian Federation from year to year. Thus, seropositive horses have been found in more than 25 regions of Russia from 2011 to 2015 [21-23].

Despite the fact that EIA AGID positive animals are registered in the Russian Federation regularly, the strain 3 isolated in Zaporozhye in 1967 by K.P. Yurov is the only known EIA virus in our country. Later, this strain was adapted to cell cultures and used as a culture called 3-K-VNIITIBP-VIEV (collection of the All-Russian Research and Technological Institute of Biological Industry, Moscow Province).

Importantly, the EIA virus molecular epizootiology has not yet been investigated in Russia, while the molecular genetic assessment of the EIA virus isolates' diversity and phylogenetic analysis are necessary to understand the specificity of this virus onset and circulation in our country. The lack of such data complicates the EIA epizootic situation. In addition, the study of the diversity of Russian EIA virus isolates will contribute to understanding its world population structure.

Our purpose was to develop the PCR-based test systems which make it possible not only to identify the EIA virus and perform its genome sequencing and genome fragment analysis, but also to study molecular genetic characteristics and phylogenetic links between the known and newly identified isolates circulating in Russia.

Technique. The only known domestic virus strain EIA 3-K-VNIITIBP-VIEV (All-Russian Research and Technological Institute of Biological Industry, Moscow Province) and two isolates identified in monitoring studies in the territory of the Russian Federation, the Nizhny Novgorod-2011 and Omsk-2012 (collection of the All-Russian Research Institute of Veterinary Virology and Microbiology), were studied.

Multiple alignment and estimation of primers for sequencing were performed by the BioEdit v. 7.0 (http://www.mbio.ncsu.edu) and Oligo v. 6.0 (Molecular Biology Insights Inc., USA) software package using the nucleotide sequences of the EIA virus various strains and isolates published in the GenBank database (the National Center for Biotechnology Information — NCBI).

Nucleic acids were isolated from biological material analyzed according to the method based on the use of Trizol LS commercial reagent (Invitrogen, USA) according to manufacturer's recommendations.

To accumulate specific products for further sequencing, we performed two round (nested) PCR without reverse transcription with the primers described by S. Capomaccio et al. [13] (for the first PCR round) and with original primers (for the second PCR round). The nested PCR option and temperature regime

optimization were performed in a Gradient Palm Cycler thermal cycler (Corbett Research, Australia).

The reaction products were separated by electrophoresis in 1.5 % agarose gel containing 0.001 % ethidium bromide. Specific amplification products were extracted from agarose gel using commercial AxyPrep DNA Gel Extraction Kit (Axygen Scientific Inc., USA) and QIAquick Gel Extraction Kit (Qiagen N.V., Germany).

Sequencing of amplified DNA fragments according to Sanger was performed in an Applied Biosystems Genetic Analyzer 3130 automatic analyzer (Applied Biosystems, USA) using Big Dye v. 3.1. Terminator and 5×Sequencing buffer (Applied Biosystems, USA) reagent kits in accordance with the manufacturer's instructions.

Phylogenetic analysis of nucleotide sequences was performed by the Neighbor-Joining algorithm using numerical resampling (resampling generation methods) and the MEGA v. 5.0 [24].

Results. The *gag* gene encoding the major matrix and capsid proteins is considered the most important marker for the study of pathogenicity and genetic diversity of EIA virus strains, which is the reason for its often use in molecular genetic studies [13, 14, 17]. Therefore, to assess the phylogenetic association between the strains identified within the territory of Russia, the foreign strains, and EIA virus isolates the genomes of which are represented in the GenBank, we identified the nucleotide sequence of this gene.

To amplify the *gag* gene fragment, primers described by S. Capomaccio et al. were used [13]. These primers (EIAV 1B and EIAV 5) flank the viral genome region of 2233 bp which includes the *tat* gene region from exon 1, a complete *gag* gene and the initial portion of the *pol* gene nucleotide sequence. PCR was performed at a temperature gradient of 50 to 59 °C, and the strain 3-K-VNIITIBP-VIEV nucleic acid was used as positive control. However, the analysis of the resulting amplified fragments demonstrated that the primers used can work at very low annealing temperature only (50-54 °C) and give a number of non-specific products in the electrophoresis.

To increase the yield of a particular product, we estimated primer oligonucleotide sequences for the second nested PCR round. These primers (EA Fw/N2 and EA Rev/N2) flank *gag* gene region of 1018 bp inside the fragment which, in turn, is flanked by the EIAV 1B and EIAV 5 primers. To perform PCR with the primers estimated, we optimized the mixture composition, the concentration of primers and dNTPs, the amount of polymerase added, and the temperature and time modes.

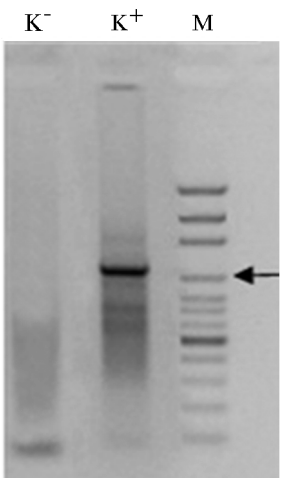

Fig. 1. Electrophoregram of the amolification products of the *gag* gene region (1018 bp) of the equine infectious anemia virus in the second round of PCR with estimated oligonucleotide primers: K^- — negative control, K^+ — strain 3-K-VNIITIBP-VIEV; M — marker of 100 bp molecular weight (Ladder DNA Marker, Axygen, USA). The arrow marks the fragment of 1000 bp.

Within 51 to 60 °C, the 56 °C was optimal for EA Fw/N2 and EA Rev/N2 hybridisation (Fig. 1). The mixture for amplification consisted of 2.0 μl of primers (10 pmol of each primer, the EIAV 1B and EIAV 5 for the first round, EA Fw/N2 and EA Rev/N2 for the second round); 1.0 μl of dNTPs (Fermentas, Latvia) (10 mM); 5.0 μl of 5× PCR buffer from the AmpliSens Blue reagent kit (Russia); 0.4 μl (2.5 U) of Taq DNA polymerase (Eurogene, Russia); 8.0 μl of studied nucleic

acid sample; and deionized water to 25 μl final volume. In the nested PCR, the amplification was performed at 52 °C in the first round (with EIAV 1B and EIAV 5 primers), and at 56 °C in the second round (with EA Fw/N2 and EA Rev/N2 primers). Primers EA Fw/N2 and EA Rev/N2 were also used for sequencing the amplified DNA fragments.

Optimized two round PCW protocol made it possible to proceed to the main study purpose and to find out, for the first time, the molecular and genetic characteristics of strain 3-K-VNIITIBP-VIEV and two EIA virus isolates (Nizhny Novgorod-2011 and Omsk-2012) identified at the disease outbreaks in the Russian Federation [25] based on the analysis of nucleotide sequences of a *gag* gene 1018 bp region.

When comparing genome regions of the domestic strain and isolates with those of the reference foreign strains from GenBank with the use of BioEdit v. 7.0, numerous nucleotide substitutions were identified. Based on multiple alignment and the EIA virus nucleotide sequences obtained and deposited by us in GenBank, a dendrogram was constructed by MEGA v. 5.0 software showing phylogenetic relationships of compared isolates and strains (Fig. 2).

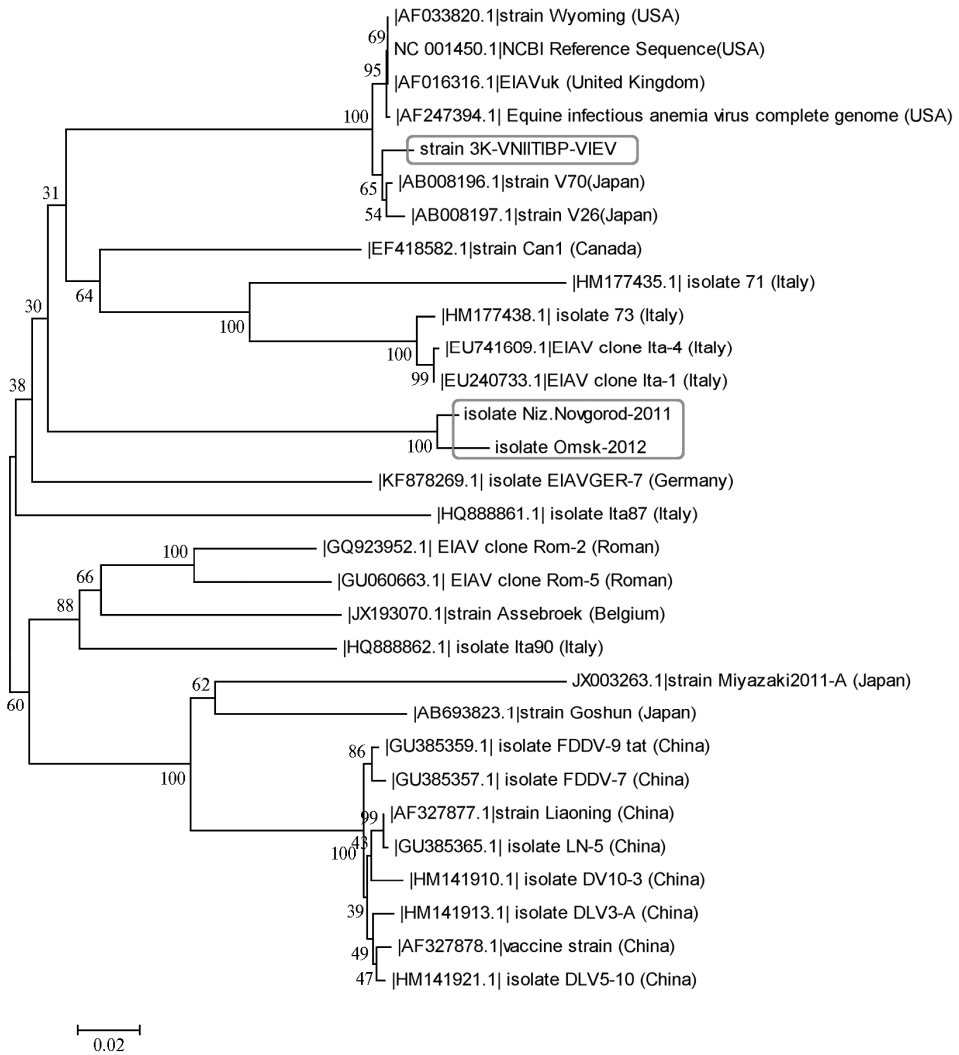

Fig. 2. Dendrogram of phylogenetic relationships between various strains and isolates of equine infectious anemia virus. Constructed based on the analysis of *gag* gene fragment (1018 bp) nucleotide sequences portions using the Neighbor-Joining algorithm and MEGA v. 50 software.

As seen in the dendrogram, strain K-3-VNIITIBP-VIEV is included into the same cluster as strains V70 and V26 (with the nucleotide identity of 98 %). According to published data, strains V70 and V26 were isolated in Japan by passaging North American $EIAV_{Wyoming}$ strain on naturally susceptible animals, and belong to the same genetic group [8, 9] with the intragroup differences not exceeding 5 %. Nucleotide sequence homology of the *gag* gene regions in strains 3-K-VNIITIBP-VIEV and $EIAV_{Wyoming}$ was also 98 %.

The isolates identified within the territory of Russia from 2012 to 2014 (Nizhny Novgorod-2011 and Omsk-2012) proved to be the phylogenetically closest ones to the group of European origin. The highest homology in the *gag* gene 1018 bp region was observed between the Russian isolates studied and German (83 % for EIAVGER-7 isolate) and Italian samples (82 % for Ita87 isolate).

Nucleotide sequences of the *gag* gene regions of strain 3-K-VNIITIBP-VIEV and Nizhny Novgorod-2011 EIA virus isolate have been deposited in GenBank as KM202106 and KM248275, respectively.

Thus, the original primers have been estimated and the nested PCR conditions have been optimized, allowing amplification of a specific equine infectious anemia virus fragment (1018 bp) in the *gag* gene region which is widely used to assess the genetic diversity and relatedness of isolates. For the first time, we have identified a fragment of genome nucleotide sequences in a domestic EIA virus strain and two recent isolates identified in the territory of Russia. The results of sequencing and phylogenetic analysis allowed assigning strain 3-K-VNIITIBP-VIEV to a group of North American origin. In addition, we have found out the highest homology between the Nizhny Novgorod-2011 and Omsk-2012 isolates, and the isolates of European origin.

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