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Genotyping Of Isolates Of Bovine Leukemia Virus As The Basis Of A Multiplex Test System For Early Diagnosis.

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ABSTRACT

Studied the genetic variability of the virus bovine leukemia (VBL) circulating in the Stavropol Territory. Based on current international classification of isolates VBL related to 4 and 6 to genotype. In addition, the selected virus isolate that is located remotely from the clusters of isolates of all known eight genotypes, which allowed to classify it as atypical. During the sequencing of the env gene area of isolates of provirus VBL allocated to this area, established the presence of 31 point mutations in the examined locus, of which 11 are significant. The results of genotyping analysis allowed a selection of target of target, to carry out the design of primers and probes that formed the basis of multiplex PCR-test-systems for indication and identification of clinically relevant genotypes VBL.

Keywords: the virus of bovine leukemia, polymerase chain reaction, sequencing, phylogenetic analysis, genotype, diagnosis of bovine leukemia, a test system for bovine leukemia.

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INTRODUCTION

In Russia, according to official studies of the Federal Service for Veterinary and Phytosanitary Surveillance, published in 2015, in the blood of every cow detected by the genome of the pathogen leukemia.

The OIE has included Russia in the category of dysfunctional leukemia among the countries of the Eurasian economic Union. The infection rate of cows varies from 30 to 70% depending on the region.

In 2011 was accepted by the technical regulations of the Customs Union "On safety of food products", not allowing the use of milk from infected cows leukemia, after a transitional period, namely from 1 January 2016. However, the industry was not ready for the requirements of technical regulations and commencement documents any trying to push.

In connection with the critical situation on leucosis of cattle in the country the greatest scientific interest are the questions of the genetic diversity of the pathogen, its mutational variability and the development of effective preventive, diagnostic and health measures to combat the disease.

To analyze the genetic variability of the leukemia virus in the Stavropol region, we carried out sequencing and subsequent phylogenetic analysis of the isolates VBL circulating in the area.

MATERIAL AND METHODS

As a clinical material, whole-blood samples of 29 cattle of black-and-white Holsteinized and red steppe breeds over the age of four years were used out of 4 dysfunctional leukemia farms in the Stavropol Territory.

Genomic DNA of cattle was extracted by nucleosorption using commercial DNA-sorb-B reagent kits (Federal Budget Institution of Science "Central Research Institute of Epidemiology", Rospotrebnadzor, Russia) according to the manufacturer's instructions. Amplification of the fragment of the proviral gene env VBL by PCR was carried out in 2 rounds ("nested" PCR) using primer pairs ENV1 (5032-5053) -ENV2 (5629-5608) and ENV3 (5099-5120)-ENV4 (5542-5521). After the second round, a 444 bp fragment was synthesized [1].

Analysis of the nucleotide sequences of the PCR products was carried out by Sanger sequencing using the ABI 3500 Genetic Analyzer ("Applied Biosystems", USA).

To determine the subspecies of VBL circulating in the territory of the Stavropol Territory, sequencing of the highly conserved fragment of the env gene of Stavropol isolates with a length of 444 bp was performed. As a reference from the international Genbank database, the nucleotide sequence of the isolate HQ902258.1 from Belorussia, closest in structure to the studied locus, was chosen (similarity level of 99%).

The data was processed using Sequencing Analysis Software v 5.4 ("Applied Biosystems", USA), FinchTV 1.4.0 ("Geospiza", USA), Chromas 2.3.3, BLAST (NCBI) algorithm, CLC Sequence Viewer corresponding to each stage [2].

RESULTS AND DISCUSSION

In the course of the studies, 31 nucleotide substitutions were detected in the analyzed locus, which in 11 cases led to missense mutations, of which 2 transversions and 9 transitions were distinguished. The most interesting is the mutation A → G at position 104, accompanied by a corresponding amino acid substitution (N → D). It is characteristic for the nucleotide sequences of the env gene of all the studied isolates of the Stavropol Territory.

Comparative analysis of the nucleotide sequence of the DNA locus env of one isolate isolated in the Trunovskoye district farm made it possible to identify five large specific deletions uncharacteristic for other samples and reference strains, which was the basis for classifying this isolate as atypical.

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The construction of the phylogenetic tree on the basis of the studied sequences of the env gene fragment allowed the typing of these VBL isolates in accordance with the modern international classification proposed by M. Rola-Luszchak (2013) [3].

The structure of the dendrogram makes it possible to clearly distinguish two branches of isolates of the leukemia virus circulating in the territory of the Stavropol Territory (Figure 1). One branch forms 96.55% of the samples, 27 of them belong to a large group, which includes a strain from France (M35238) and a reference strain from Belarus (HQ902258.1), representing the genotype 4 (the degree of kinship is 98-99%). In the structure of this branch it is possible to identify several groups and their individual clades, represented mainly by isolates from specific livestock farms. In addition, one isolate (9157_U) from the Novoaleksandrovsky district is a separate treasure with an isolate from Brazil AY185360, which probably indicates its genetic similarity to the representative of genotype 6 [4].

Atypical isolate (29260) from the economy of the Trunovsky district formed a separate cluster on the dendrogram, isolated from the other isolates and reference samples considered earlier, which is a consequence of the unique primary structure of the DNA locus being studied, in which at least 65% of the nucleotide residues are deleted.

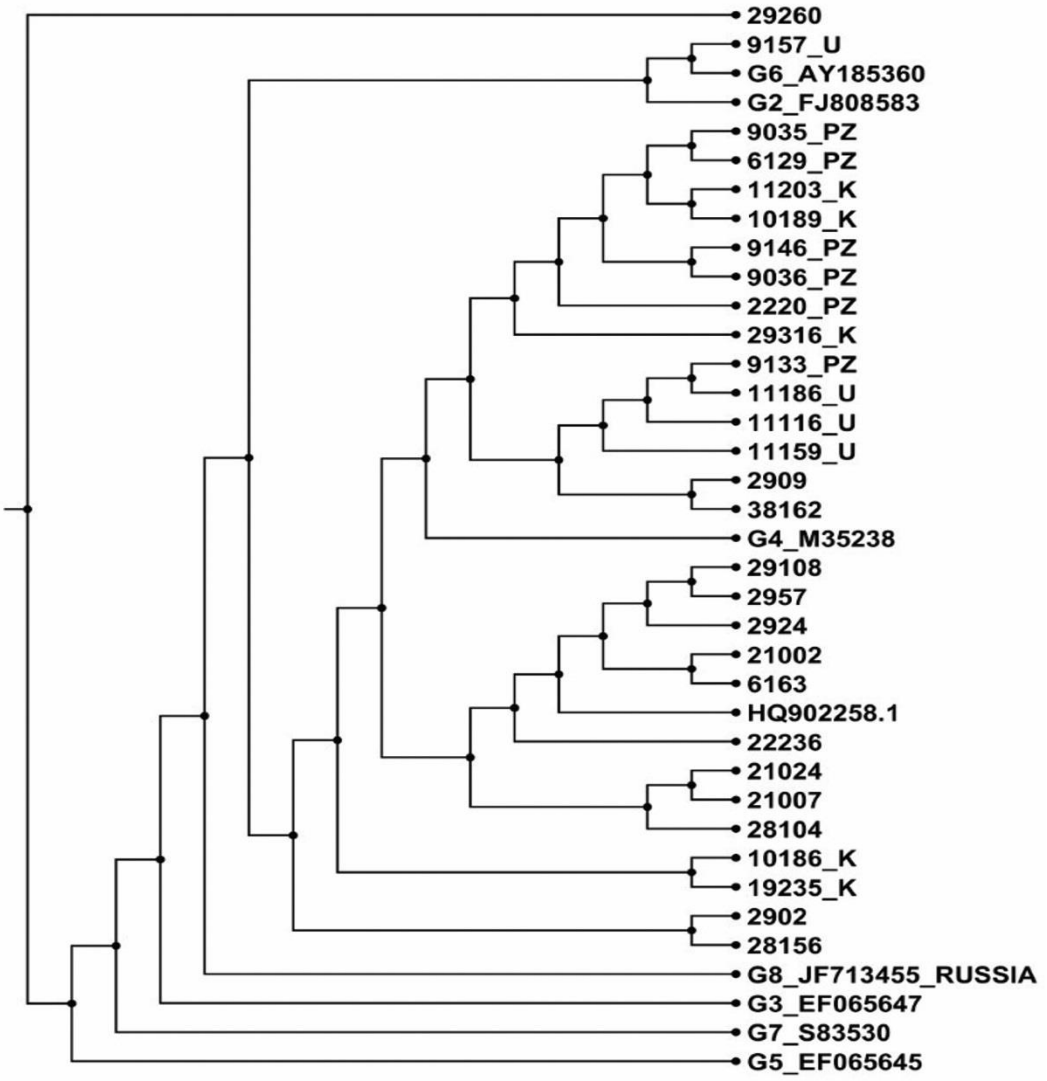

Figure 1: Cluster analysis of 37 strains representing the reference (n=8) strains and studied isolates (n=29) based on the results of sequencing of the locus env leukemia virus of cattle

When studying the morphology of blood cows infected with a leukemia pathogen with mutational changes in the env gene locus, it is interesting that 100% of the VBL carriers of genotype 4 have lymphocytolysis from moderate to severe degree. In 38.5% of cases, cells with an irregular, often polymorphic nucleus - "Reader" forms of lymphocytes, in 53.8% - a significant number of monomorphic monotypic lymphocytes with a hyperchromic nucleus, in 23.1% blast and binuclear lymphocytes, in 38.5% % - active apoptosis of lymphocytes, 7.7% - cytomegal lymphocytes and plasma cells in peripheral blood.

Analysis of the data showed that the VBL circulating in the territory of the Stavropol Territory of Russia is evolutionarily close to the cluster of isolates assigned to genotypes 4 and 6, which corresponds to the data of a number of scientists.

The results of the work, namely ten nucleotide sequences of the fragment of the proviral gene env of VBL isolates circulating in the Stavropol Territory, were deposited in the international Genbank database (NCBI) with assignment of registration numbers KP308390, KR007590- KR007598.

Analysis of the morphological picture of blood suggests that the formation of hemoblastosis in patients with enzootic leukemia of animals is associated with an increase in the oncogeneity of VBL, which is probably associated with mutational changes in the locus of the env gene, which can also cause a significant risk of early manifestation of enzootic leukemia with a high percentage (53.8 %) transition of the disease from the latent phase to the hematological stage with more pronounced pathological changes.

In this connection, it became necessary to develop a new highly sensitive test system for the diagnosis of leukemia of cattle using polymerase chain reaction with hybridization-fluorescent detection in real time, which allows not only to detect the presence of the virus, but also to genotyped it.

At the moment, work is underway to create such a test system. Based on the data of the bioinformation analysis, SNP targets were selected for differentiating clinically significant genotypes of the leukemia virus within the framework of one diagnostic test, selection and design of an optimal primer-probe system were performed, and laboratory testing was conducted.

CONCLUSION

Upon completion of the research, it is planned to create a competitively capable product that allows to identify with the greatest accuracy leukemia-infected animals, including those with an atypical form, to develop an individual strategy for managing sick animals, depending on the oncogeneity of the pathogen, which, in the final analysis, will lead to a significant reduction in the health of the herd (up to 1 year with 10% infection), improving its quality and preventing repeated outbreaks of leukemia.

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