DETECTION OF AVIAN LEUKEMIA VIRUS SUBGROUP K IN RUSSIA AND ITS MOLECULAR GENETIC ANALYSIS

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Abstract

The Avian leukemia virus (ALV) belongs to the genus Alpharetrovirus (Retroviridae). The genome of the virus is a single-stranded RNA of more than 7,000 nucleotides in length. The ALV subgroups A, B, C, D, J, K and E are specific viruses of chicken. ALV classification is based on the type-specific envelope protein antigens, GP85. ALV is widely spread all over the world, causes various diseases, reduces productivity and leads to huge damage to poultry industry. The viruses of subgroup K were first discovered in Asian countries. Studies of the prevalence of ALV of subgroup K are few and have not been conducted in Russia before. Our goal was to study the spread of the ALV of this subgroup to the chickens of Russian poultry farms using a test system designed to identify the genome of the ALV subgroup K by real-time PCR and analysis of the properties of the ALV of subgroup K. The test of the real-time PCR test system was carried out on 5292 DNA samples of chickens of domestic broiler meat type of one of the farms in the Moscow Province and in chickens of meat and egg breeds from various regions of Russia. The ALV-K-specific gene gp85 sequences were found in 177 (3.3 %) broilers of one of the farms in the Moscow Province. Sequencing of gp85 gene fragments revealed the presence of two groups of different ALV subgroup K in the chickens of this farm: one had 96 % similarity to the ALV subgroup K strains Oki 009, GDFX0601, GDFX0602, GDFX0603, Km_5845 etc., and the other - up to 100 % similar to ALV subgroup K strain TW-3593. Using the reference sequence of the ALV subgroup K strain GD14LZ (KU605774), 22 full-length genomic sequences, belonging to ALV subgroup K, with 95 to 100 % similarity to the GD14LZ gp85 gene sequences were detected in GenBank. The construction of phylogenetic trees based on the gp85 gene sequences showed that subgroup K viruses form a separate group, distinct from the rest of the viruses, which can be divided into four clusters based on differences in long terminal repeats. The subgroup K viruses have a set of four different 3´UTR sequences belonging to both the pathogenic ALV subgroup J and the less pathogenic ALV subgroup E, and there are also ALV K and Oki, the ALV-K-specific 3´UTRs. In addition to the Moscow region, ALV subgroup K was found in the Kaliningrad, Leningrad, Sverdlovsk, and Novgorod regions of Russia. Thus, the...
distribution of ALV subgroup K is not limited to the countries of Asia. Scientific publications show that ALV subgroup K can cause gliomas and myocarditis. Literature data allows us to say that ALV subgroup K can be pathogenic and these viruses need a control program, because even the subclinical form of exogenous and endogenous ALV can lead to large economic losses.

**Keywords:** avian leukosis virus subgroup K, real time PCR, ALV subgroup K detection

Avian leukosis virus (ALV) belongs to the *Alpharetrovirus* genus of the *Retroviridae* family. The virus genome has the form of a single-stranded RNA of more than 7000 nucleotides in length. The A, B, C, D, J, K and E subgroups of ALV are specific for chickens. These subgroups differ with their antigenic regions of the GP85 coat protein [1-3]. ALV can cause not only lymphoid and myeloid leukosis, but also neoplasms of other tissues [4-6] including gliomas and neurofibrosarcomas [7, 8]. The virus subgroup can be identified basing on the analysis of the nucleotide sequence of the GP85 coat protein gene [3, 9, 10]. Depending on the way of infecting the host cells, ALV is divided into exogenous [1, 4] and endogenous viruses. The viruses of A, B, C, D, J and K subgroups belong to exogenous ones and are more pathogenic than the endogenous virus of E subgroup which has no or weak pathogenicity. The genome of endogenous viruses is integrated into the host’s genome and is transmitted vertically like the rest genes of the host. Exogenous viruses are able to spread congenitally by infecting hens and the embryos form infected hens (a form of vertical transmission). Endogenous ALV can sometimes behave as exogenous ones infecting chickens horizontally. Usually, the chicken mortality caused by ALV infection does not exceed a few percent, but for active form of infection, the mortality rate may amount to more than 20% [9]. ALV is widely spread all over the world and can cause various pathologies, reducing bids’ performance and leading to huge damage to commercial poultry.

The ALV of K subgroup was recently found in Asia, i.e. in China [3, 10, 11], Japan [8], and Taiwan [12]. The pathogenicity of these isolates is unclear. There is a danger of recombination of the K subgroup ALVs with the viruses of other subgroups, in particular with ALV-J that may lead to the emergence of a new subgroup of ALV with higher pathogenicity [10, 11]. The studies focusing the prevalence of the K subgroup of ALV are few and have not been conducted in Russia before [13].

This article reports the first identification of the K subtype of ALV in Russian industrial populations of chickens and presents the results of its molecular-genetic studies.

Our goal was to detect the K subgroup of ALV in poultry farms in Russia with using the developed testing system based on the real-time PCR (q-PCR) and the subsequent analysis of the properties of the K subgroup of ALV.

**Techniques.** The primers and probe for the RT-PCR and Sanger sequencing were synthesized in the Syntol LLC. They were selected so as to amplify the DNA of K subgroup ALVs without amplifying the known endoviruses. The carboxyfluorescein (6FAM) was used as a fluorescent tag, BHQ-1 dye was a fluorescence quencher, and phosphate (p) was used for the 3’ terminal modification of the probe.

The following primers and probe were used to identify the K subtype of ALV by the RT-PCR method:

- **ALVKF** (5’→3’) — CGGAGCATTGACACGCTTTCAGA,
- **ALVKR** (5’→3’) — GTGGTTGCGGAGGA,
- **KPL** (5’→3’) — (6FAM)CCACCTCGTGAG(dT-BHQ-1)TGCGGCC-p.

The length of the synthesized amplicon is 72 bp, the fragment is a part of the *gp85* gene encoding the GP85 coat protein.

The primers which have been used for PCR and sequencing the DNA of
the K subtype of ALV:

ALVKF (5’→3’) — CGGAGCATTGACAAGCATTTCAGA,  
SEQA-KR (5’→3’) — CGCGATCCCCACAAATGAGGAAA.

The length of the amplification product (part of the gp85 gene encoding the GP85 coat protein) is 466 bp.

Typing of 3’ terminal fragments of gp85 genes and of the variants of long terminal repeats (LTRs) has been performed using three reverse primers and SEQKF universal primer:

SEQKF (5’→3’) — GGCCGTTCATTTGCTGAAAGGA,  
SEQKRKm (5’→3’) — CAGGCTAGGCACTTAAGTACAACA,  
SEQKRJ (5’→3’) — GGGCACTTAAATACAGTATCTCTG,  
SEQKROKI (5’→3’) — CAATCAGCATGCGCCACGATGAA.

To amplify the 3’ terminal fragments of the gp85 gene and of the LTRs similar to those of the endovirus, we used the special pair of primers which preclude the amplification of the sequences of DNA of the E subgroup of ALV:

SEQKF12 (5’→3’) — GTGGCTCCTCCTCCGCCGCAA,  
SEQKREV (5’→3’) — GCAGCTTATATAATCGTGCATAGC.

The tryout of the testing system was performed on 5292 DNA samples of chickens of broiler-type meat cross from a farm of the Moscow region.

DNA was extracted from feathers using M-Sorb kit (Syntol LLC, Russia, a Savraska-02 robotized complex for molecular-genetic researches, Syntol LLC, Russia). A 0.3-0.5 cm fragment of feather was put in a 1.5 ml test tube, then 400 μl of the lysing solution was added and incubated at 60 °C for 20 min with mixing. The lysate was precipitated in the high-speed Cyclotemp-902 microcentrifuge (Cyclotemp CJSC, Russia) for 3 min at 13000 rpm. The supernatant was transferred to a 1.5 ml test tube and the extraction was continued according to the standard protocol for the M-Sorb kit.

The mode for the PCR performing was universal, 1.5 μl of the extracted DNA was taken for the reaction. The primers concentration in the reaction mixture was 450 nM, the probe concentration was 150 nM. RT-PCR (detection channel FAM) was performed (an ANK-48 device, Analytical Instrumentation Institute RAS, Russia) according to the following program (45 cycles): denaturation at 93 °C for 10 s, annealing at 60 °C for 30 s. To amplify DNA, the reaction mixture (10 μl) for the RT-PCR was used (Cat. No. M-428, Syntol LLC, Russia).

The specificity of RT-PCR was confirmed by sequencing of the amplification products obtained with ALVKF and SEQA-KR primers (Nanofor 05 genetic analyzer, Analytical Instrumentation Institute RAS, Russia).

In phylogenetic analysis and selection of the specific DNA regions for typing the K subgroup of ALV and sequencing, the genomic sequences of ALV strains of different subgroups were used: A subgroup — MQNCSU-A (DQ365814); B subgroup — Schmidt-Ruppin B (AF052428); C subgroup — Prague C (J02342.1); D subgroup — Schmidt-Ruppin D (D10652); E subgroup — ev-1 (AY013303); J subgroup — HPRS103 J (Z46390); K subgroup — Km_6222 (AB764103), Km_5943 (AB669897), Km_5845 (AB670314), Km_5844 (AB670312), Km_6202 (AB764101), Km_5892 (AB682778), Km_6181 (AB764100), Km_6349 (AB764106), Km_6249 (AB764104), Km_6343 (AB764105), Sp-53 (AB617820), SD110503R (KF738251), Sp-40 (AB617819), JS14CZ02 (KY490696), GDFX0601 (KP686142), GDFX0602 (KP686143), TW-3593 (HM582658), GD14LZ (KU605774), JS11C1 (KF746200), GDFX0603 (KP686144), JS14CZ01 (KY490695), Oki 009 (AB669433) (in the parentheses are the numbers of the GenBank). The conservative regions of the ALV genome, which are specific for the K subgroup, were detected (ClustallW and BLAST.
the K subgroup, have been used for the amplifying and sequencing the DNA. When designing of the system, the synthetic fragment of DNA corresponding to the estimated amplicon for the ALV of K subgroup was the positive control. The analytical sensitivity of the system was evaluated in the test with dilutions of the positive control. It amounted to 100 copies of the ALV genome of K subgroup or approximately 70 genome equivalents per 1 μl of the initial sample. The solution not containing the dilutions of the synthetic amplicon was the negative control.

Using the developed testing system, 5292 DNA samples from broilers of a farm in the Moscow region have been examined. The specific sequences of the gp85 gene of the K subgroup ALVs were found in 177 birds that amounted to 3.3% of the total number. The samples collected in other regions of Russia have been also analyzed. As the result, the genomic sequences of the K subgroup ALVs were also found in Kaliningrad region (3 samples), Leningrad region (2 samples), Sverdlovsk region (5 samples) and Novgorod region (3 samples). Considering the fact that the poultry of one of the poultry farms of the Kaliningrad region, where the ALVs of K subgroups have been detected, has been supplied from Germany, the geography of distribution of this ALV type in Europe is probably not limited to Russia.

To confirm the specificity of the testing system, the fragments of gp85 gene were sequenced using the ALVKF and SEQA-KR primers and DNA of 12 chickens from a farm of the Moscow region. The analysis revealed two variants of genomic sequences of the K subgroup ALV. In the first group, the virus had 96% similarity with the Oki 009, GDFX0601, GDFX0602, GDFX0603, Km_5845 and other ALV strains, in the second group the similarity was up to 100% with the TW-3593 strain. The sequencing showed that both variants of examined ALV have the LTRs similar to those of the E subgroup of ALV.

In analysis of geographic prevalence and relation of K subgroup ALV with the chicken diseases, we used the KU605774 sequence of the GD14LZ isolate genome of the K subgroup ALV as the reference sequence based on the GenBank data [3]. The presence of sequences similar to the GD14LZ genome has revealed the countries in which the ALV of K subgroup is widespread. A number of full-length genomic sequences of ALV with 95-100% similarity of the gp85 gene sequences with the gp85 gene sequence of the GD14LZ isolate has been found in the GenBank database. The variability of the viral genome of the J subgroup ALV is very high and may reach 94.9% within one organism [14]. The pool consisting of 22 full-length genomic sequences of ALV, which presumably belong to the K subgroup, was formed and analyzed (Table, Fig.).

The construction of phylogenetic trees based on the nucleotide sequences of gp85 gene fragments showed that the ALV K subgroup forms a separate group which is different from the rest viruses (see Fig., A). The analysis of the 3'-UTR sequences of ALV K subgroup demonstrates viral heterogeneity and the fact that the K subgroup can be divided into four clusters (see Fig., B). The ALV K subgroup has four different 3'-UTR regions (see Table) similar to 3'-UTR of both the pathogenic strains of ALV J subgroup and less pathogenic E subgroup. There are also 3'-UTRs specific for the K subgroup (ALV K and Oki).

Different 3'-UTRs could be obtained as the result of recombination with other viruses, for example, the virus carrying gp85 gene from the ALV A subgroup and the LTR from ALV J subgroup is known [15]. The ALV J subgroup
presumably results from recombination between endogenous and exogenous forms of the virus [16].

Pathogenic properties and prevalence of K subgroup avian leukosis virus isolates described in the literature and represented in the GenBank (NCBI)

<table>
<thead>
<tr>
<th>Isolate (area)</th>
<th>No. in GenBank</th>
<th>Author (reference)</th>
<th>Type of 3'-UTR</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD110503R (China)</td>
<td>KF738251</td>
<td>Chen J. n.p.</td>
<td>ALV J</td>
<td>Unknown</td>
</tr>
<tr>
<td>Sp-40 (Japan)</td>
<td>AB617819</td>
<td>Nakamura S. et al. [8]</td>
<td>ALV J</td>
<td>Glioma</td>
</tr>
<tr>
<td>Sp-53 (Japan)</td>
<td>AB617820</td>
<td>Nakamura S. et al. [8]</td>
<td>ALV J</td>
<td>Glioma</td>
</tr>
<tr>
<td>Km_6222 (Japan)</td>
<td>AB764103</td>
<td>Nakamura S. n.p.</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>Km_6343 (Japan)</td>
<td>AB764105</td>
<td>Nakamura S. n.p.</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>Km_6181 (Japan)</td>
<td>AB764100</td>
<td>Nakamura S. n.p.</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>Km_5892 (Japan)</td>
<td>AB682778</td>
<td>Nakamura S. et al. [17]</td>
<td>ALV K</td>
<td>Myocarditis</td>
</tr>
<tr>
<td>Km_6249 (Japan)</td>
<td>AB764104</td>
<td>Nakamura S. n.p.</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>Km_6349 (Japan)</td>
<td>AB764106</td>
<td>Nakamura S. n.p.</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>Km_6222 (Japan)</td>
<td>AB764103</td>
<td>Nakamura S. n.p.</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>Km_5844 (Japan)</td>
<td>AB670312</td>
<td>Ochi A. et al. [18]</td>
<td>ALV K</td>
<td>Glioma</td>
</tr>
<tr>
<td>Km_5845 (Japan)</td>
<td>AB670314</td>
<td>Ochi A. et al. [18]</td>
<td>ALV K</td>
<td>Astrocyte proliferation</td>
</tr>
<tr>
<td>Km_5943 (Japan)</td>
<td>AB669897</td>
<td>Ochi A. et al. [18]</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>JS11C1 (China)</td>
<td>KF746200</td>
<td>Cui N. et al. [10]</td>
<td>ALV K</td>
<td>Unknown</td>
</tr>
<tr>
<td>JS14CZ01 (China)</td>
<td>KY490695</td>
<td>Shao H. et al. [11]</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
<tr>
<td>TW-3593 (Taiwan)</td>
<td>HM582658</td>
<td>Chang S.W. et al. [12]</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
<tr>
<td>GDFX0601 (China)</td>
<td>KP686142</td>
<td>Jianyong H. n.p.</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
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<td>GDFX0602 (China)</td>
<td>KP686143</td>
<td>Jianyong H. n.p.</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
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<td>GDFX0603 (China)</td>
<td>KP686144</td>
<td>Jianyong H. n.p.</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
<tr>
<td>JS14CZ02 (China)</td>
<td>KY490696</td>
<td>Shao H. et al. [11]</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
<tr>
<td>GDI4LZ (China)</td>
<td>KU605774</td>
<td>Li X. et al. [3]</td>
<td>ALV E</td>
<td>Unknown</td>
</tr>
<tr>
<td>Oki 009 (Japan)</td>
<td>AB669433</td>
<td>Ochi A. et al. [18]</td>
<td>Oki</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Note: “n.p.” means the unpublished data (deposited in the GenBank).

The analysis of the literature shows that the ALV K subgroup can cause gliomas and myocarditis [8, 17, 18] which are atypical for the ALV pathology. In the case of avian leukosis virus, the mechanism of pathogenesis is not yet fully studied. It is assumed that the high variability of retroviruses may contribute to their virulence and pathogenesis [19]. In the LTR areas of some ALV strains, there is the E element which is not an oncogene but increases the pathogenicity of the viruses [20]. Some of the studied sequences of the ALV K subgroup have a similarity to the E element. The example is SD110503R (KF738251) strain from China (nothing is known about the pathogenicity of this strain because only the genomic sequence in the GenBank has been published). Two ALV strains causing glioma are described in Japan, these are Sp-40 (AB617819) and Sp-53 (AB617820). Their genomes contain a small fragment of 27 nucleotides in length which is similar to the E element [8] (see Table). Insertional mutagenesis is a mechanism in infection by avian leukosis virus [21, 22]. Also, the expression of the host cell genes may change due to additional transcription of ALV LTR [23, 24]. Much in the infection expression mechanism is still unclear because the spectrum of diseases caused by ALV is quite wide. For example, it is assumed that in the hemangiomatosis progression the leading role belongs to the insertion mutagenesis with changing of the met gene expression [21], other authors point out the importance of the participation of GP85 coat protein in the disease progression [24]. There is the evidence that the coat protein is the main determinant when lymphoid and myeloid leukosis [25]. It may be assumed that the presence of ALVK subgroup reduces the productive performance of poultry. Thus, it is believed that the ALVs having the LTRs similar to the LTRs of the ALV E subgroup do not have any noticeable pathogenicity. However, the metagenomic analysis of the possible infectious agent which has caused up to 20% loss of the chicken population [26] showed that the nucleotide sequences of this virus express 100% similarity with the gp85 gene and the LTR of the ALV strain TW-3593 which has the LTR of the ALV E subgroup.
Phylogenetic trees constructed for the sequences of gp85 gene of coat protein (A) and 3’-UTRs (B) of strains of different avian leukosis virus subtypes from the GenBank. See the description of these strains in the Techniques section.

The exogenous and endogenous avian leukosis viruses may cause large economic losses even in subclinical infection [6, 27]. To study the pathogenic properties of the ALV K subgroup which we have found in the broiler-type meat cross, 123 infected birds at the age of 58 days were isolated from the main herd. We have not detected any neoplastic processes in 6 birds died at the age of 156 days.

So, the real-time PCR testing system has been developed for detecting the avian leucosis virus of K subgroup. Using this test we have detected the virus of this subgroup in Russia. Thus, the prevalence of the ALV K subgroup is not limited to Asian countries. The analysis of the literature data indicates possible pathogenicity of the K subgroup ALV, therefore, the program for its monitoring and control is needed.

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