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MULTIPLEX MULTILOCUS REAL TIME PCR FOR ANALYSIS AND CONTROL OF AVIAN LEUKOSIS VIRUS SUBGROUPS A, B, J AND K IN RUSSIA

A.M. BORODIN[1, 5], *Ya.I.* ALEKSEEV[2, 3], N.V. KONOVALOVA[3], E.V. TERENTYEVA[3], N.Yu. SEROVA[4], D.N. EFIMOV[5], Zh.V. EMANUILOVA[5], S.V. SMOLOV[5], O.A. OGNEVA[5], V.I. FISININ[6]

[1]*Non-profit Partnership Institute of Medico-Biological Research*, 10, ul. Studenaya, Nizhnii Novgorod, 603000 Russia, e-mail Aborodinm@sinn.ru;
[2]*Institute for Analytical Instrumentation RAS,* 31-33, ul. Ivana Chernyh, St. Petersburg, 198095 Russia, e-mail jalex@syntol.ru;
[3]*LLC Syntol*, 42, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail jalex@syntol.ru (✉ corresponding author);
[4]*All-Russian Research Veterinary Institute of Poultry Science — Branch of Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS*, 48, ul. Chernikova, St. Peterburg—Lomonosov, 198412 Russia, e-mail vnivip.lab@gmail.com;
[5]*Breeding and Genetic Center Smena*, pos. Bereznyaki, Moscow Province, 141327 Russia, e-mail Smena@tsinet.ru, dmi40172575@yandex.ru;
[6]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141315 Russia, e-mail olga@vnitip.ru, vnitip@vnitip.ru
ORCID:
Borodin A.M. orcid.org/0000-0002-1478-1261
Alekseev Ya.I. orcid.org/0000-0002-1696-7684
Konovalova N.V. orcid.org/0000-0003-4316-1077
Terentyeva E.V. orcid.org/0000-0003-2777-0948
Serova N.Yu. orcid.org/0000-0003-2121-2048
Efimov D.N. orcid.org/0000-0002-4152-2476
Emanuilova Zh.V. orcid.org/0000-0002-8855-2947
Smolov S.V. orcid.org/0000-0001-6058-3672
Ogneva O.A. orcid.org/0000-0002-8698-1975
Fisinin V.I. orcid.org/0000-0003-0081-6336
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Abstract

The avian leukosis virus (ALV) belongs to genus *Alpharetrovirus* of *Retroviridae* family and has a diploid genome consisting of a single-stranded RNA. ALV subgroups A, B, C, D, E, J and K are specific for chicken. The classification is based on differences in the viral coat protein structure. ALV, in particular ALV subgroup J, causes huge damage to industrial poultry. The gold standard for detecting ALV is virus isolation in CEFs or DF-1 cell cultures. This method has significant disadvantages, i.e. it takes 7-9 days, requires specialized facilities and equipment. Enzyme-linked immunosorbent assay based on detection of the ALV p27 group-specific antigen is the most widely used, but it also has significant deficiencies, the main of which are false positive results due to the expression of p27 by endogenous viruses and lack of sensitivity. In this study, a test system has been developed to detect exogenous viruses of the most common ALV subgroups A, B, J, and K and to control the spread of ALV. To test the developed system, we use 1200 samples of broiler DNA from a poultry farm of the Moscow Province. Analysis of the samples detected ALV subgroup J in 51 % poultry flock and ALV subgroup K in 8 % poultry flock. No viruses of subgroups A and B were found. We also analyzed 97 DNA samples from chickens from the regions of Russia, i.e. Orenburg, Chelyabinsk, Kemerovo, Tyumen, Kaliningrad, Leningrad, Sverdlovsk, Novgorod regions and Krasnodar Territory. ALV subgroups K were found in samples from the Kaliningrad, Leningrad, Sverdlovsk, and Novgorod regions, ALV subgroups A in samples from the Leningrad region, and ALV subgroups J in the Sverdlovsk and Leningrad regions. ALV subgroup B has not been identified, that is, it may indicate that this subgroup of ALV is not common in Russia at the present time. At the next stage, measures were taken to eradicate the ALV of subgroups J and K found in the broiler-type meat cross lines in one of the farms of the Moscow Province. For this, a multiplex multilocus real-time PCR test system was developed and applied for the simultaneous detection of ALV subgroups J and K. Using the proposed test system, several screening cycles of four broiler-type chicken meat cross lines with an initial total of 9029 chickens were performed. Prior to the start of the program for control and eradication of ALV, the proportion of poultry with neoplasia ranged from 17 to 26 % depending on the line of chickens (the maximum was observed in the line with the *ev21* locus). On

the 265th day after the start of the program for the control and eradication of ALV subgroups J and K, only three out of 2621 individuals (0.10 %) were diagnosed with a diagnosis of neoplasia, confirmed by positive results of real-time PCR as ALV subgroup J. Total percentage of individuals' samples containing ALV DNA of subgroups J and K in a sample of 2621 individuals at the age of 265 days were 0.67 % and 0.04 %, respectively.

Keywords: Avian leukosis virus, ALV subgroups A, B, J and K, real time PCR, ALV detection

The avian leukosis virus (ALV) belongs to genus *Alpharetrovirus* of *Retroviridae* family and has a diploid genome consisting of a single-stranded RNA. ALV subgroups A, B, C, D, E [1], J [2] and K [3] are specific for chicken. The ALV classification is based on differences in the GP85 coat protein structure [2, 3]. Exogenous ALV (subgroups A, B, C, D, J and K) spreads horizontally (bird-to-bird) and vertically (from parents to offspring through an egg). These viruses are characterized by greater pathogenicity than endogenous virus E with weak or even absent pathogenicity. Infecting an embryonic cell, endogenous viral genome builds in a host's genome. As per the Mendel's law, it is transmitted vertically [1]. ALV induces lymphoid/myeloid leukosis and other neoplasms [1]. Sometimes chicken morbidity and mortality can be 60% and > 20%, respectively [4]. ALV (subgroups A, B and J) is the most common worldwide whereas ALV-C and D are extremely rare [1]. Since 2007 China implements a National ALV Eradication Programme focused on ALV-J combating. Also, these viruses represent a critical issue in Russia. In particular, antibodies to them are detected in 70% of 223 monitored poultry farms in 46 regions, and general antibodies to ALV were observed in 90% of farms [5].

The gold standard for detecting ALV is virus isolation in CEFs or DF-1 [1] cell cultures. The method has substantial disadvantages because of long duration (7-9 days) and required sterile premises and equipment. Enzyme-linked immunosorbent assay based on detection of the ALV p27 group-specific antigen is the most common method of ALV testing. However, it also has significant disadvantages, the main of which are false positive results caused by p27 expressed by endogenous viruses [6] and lack of sensitivity [1, 7]. Moreover, endogenous viruses cause false positive results in several modern test systems based on the real-time polymerase chain reaction (RT-PCR) intended for ALV detection [6]. Sensitivity of RT-PCR test systems to detect ALV is by 15-20% higher than in cultural and enzyme-linked immunosorbent methods [8].

In the context of the study, a multiplex multilocus real-time PCR (RT-PCR) test system was developed and applied for simultaneous detection of the most common ALV subgroups (A, B, J and K), as well as for simultaneous detection of ALV-J and K.

The purpose was to apply developed test systems to detection and eradication of ALV.

Techniques. Using Blast software together with the GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and ClustallW (http://www.genome.jp/tools-bin/clustalw) databases, we selected conserved regions of ALV genome specific for subgroups A, B, J and K. Further, they were used as targets for DNA amplification and sequencing. To choose specific regions, we used reference sequences related to different ALV subgroups stored in the GenBank such as M37980, HM452341 (ALV-A); AF052428, JF826241 (ALV-B), J02342 (ALV-C); D10652 (ALV-D); EF467236, AY013303, AY013304, KC610517 (ALV-E); Z46390, JF951728, JQ935966, HM776937, JX855935, JF932002, KX058878, DQ115805, KX034517, KU997685, HM235668, HM582657 (ALV-J). Several sequences isolated in various Russian regions are presented among them (i.e., KF746200, KP686143, GD14LZ (ALV-K)). Primers and probes intended for RT-PCR and Sanger sequencing were developed on the basis of reported DNA sequences [10, 11, 15] and synthesized by

Syntol OOO. They were selected in such a way as to amplify ALV DNA without amplification of known endoviruses. 6FAM, 5R6G, 6ROX, Cy5 and Cy5.5 colorants were used as a fluorescent label in R-T PCR hybridization probes. BHQ1 and BHQ2 colorants connected to thymidine inside the hybridization probe with a linker were fluorescence quenchers. A phosphate (P) group was used for 3′-end probe modifications.

DNA was extracted from chicken feathers with a M-Sorb reagent kit (Syntol OOO, Russia). A feather 0.3-0.5 cm fragment or chicken cloaca swab was placed in a 1.5-ml tube. Then, we added 400 μl of lysis solution and incubated a sample at 60 °C for 20 min. Lysate was precipitated in a high-speed microcentrifuge (Cyclotemp-902; Cyclotemp ZAO, Russia) at 13,000 rpm for 1 min. Supernatant was transferred to a 1.5-ml tube. The isolation was proceeded according to the standard schedule of a M-Sorb reagent kit (Syntol OOO, Russia). A real-time polymerase chain reaction involved 1.5 μl of isolated DNA.

Reverse transcription was performed with OT-1 reagent kit (Syntol OOO, Russia). In this, 10 μl of nucleic acid preparation isolated with M-Sorb reagent kit was added to 25 μl of the reaction mixture.

RT-PCR was carried out with ANK-48 (Institute of Analytical Instrument-Making of the Russian Academy of Science, Russia) and DT-96 (DNK-Tekhnologii OOO, Russia) as per the program as follows: 90 °C for 30 s; denaturation at 90 °C for 10 s, annealing at 60 °C for 30 s (50 cycles). To amplify DNA, we used 10 μl of finished 2.5× M-428 RT-PCR reaction mixture (Syntol OOO, Russia). Concentrations of primers and probes in the reaction mixture were 450 nM and 100 nM, respectively.

RT-PCR specificity was verified by sequencing of amplification products obtained with ALVKF, SEQA-KR, SEQJF, SEQJR, ALVAF, SEQA-KR primers with a Nanofor 05 genetic analyzer (Institute of Analytical Instrument-Making RAS, Russia) equipped with DNK Analiz 5.0.2.3 software (Institute of Analytical Instrument-Making RAS, Russia).

Results. A multiplex RT-PCR of the most common avian leukosis virus subgroups. Although ALV-J, A and B are the most common worldwide, C and D subgroups are extremely rare. Comparatively recent [3, 13-16] ALV-K is of interest to study its spread and pathogenic properties. Even subclinical exogenous and endogenous ALVs lead to decreased poultry productivity and great economic losses [1, 17]. That is why, the multiplex test system was designed to detect the subgroup as well (Table 1). A *gp85* coat protein gene of appropriate ALV subgroups was used as a DNA target of test systems. Synthetic DNA fragments correspondent with estimated amplified ALV (A, B, J and K) genome fragments were used as positive controls (see the Table 1).

To evaluate sensitivity of the test system, we used successive 10-fold and 2-fold dilutions of positive control samples. Analytical sensitivity of the test system assessed by dilutions of a positive control whose amount was calculated as per the description [11, 12] was 25 genome equivalents (ALV-A), 10 genome equivalents (ALV-J), 25 genome equivalents (ALV-B) and 10 genome equivalents (ALV-K) per 1 μl of an initial sample.

1. Primers and probes used in the multiplex test system to detect various avian leukosis viruses (ALV)

Primers, probes	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALF	AGCACCACTGCTGCCTTGGA	62	ALV A-E
ALR	CTAGCGACCGCTCCTTCCAGA		
ALVPL	(6FAM)CGATGGGACCC(dT-BHQ1)GCCCTGC-P		

Table 1 continued

ALVAF	GCCACACGGTTCCTCCTTAGA	114	ALV A
ALVAR	CGCAGTACTCACTCCCCATGAA		
APL	(5R6G)TACGGTGG(dT-BHQ1)GACAGCGGATAG-P		
JFF1F	GCCCTGGGAAGGTGAGCAAGA	139	ALV J
JJR	GGAAATAATAACCACGCACACGA		
JNP	(6ROX)TCCTCTCGA(dT-BHQ2)GGCAGCAAGGGTGTC-P		
ALBF1	GGCCGAGGCCTCCCCGAAA	77	ALV B
ALVBR	GTCTCATTAATTTCCTTTGATTGA		
BPL1G	(Cy5)CCCATGTACC(dT-BHQ2)CCCGTGCCTTG-P		
ALVKF	CGGAGCATTGACAAGCTTTCAGA	72	ALV K
ALVKR	GTGATTGCGGCGGAGGAGGA		
KPL	(Cy5.5)CCACCTCGTGAG(dT-BHQ2)TGCGGCC-P		

2. Primers and probes used in reference test system to detect avian leukosis virus J (ALV-J)

Primers, probes	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALV-JNF	TTGCAGGCATTTCTGACTGG	214	ALV J [8]
ALV-JNR	ACACGTTTCCTGGTTGTTGC		
JCP	(6FAM)CCTGGGAAGGTGAGCAAGAAGGA-BHQ1		
H5	GGATGAGGTGACTAAGAAAG	545	ALV J [9, 10])
H7	CGAACCAAAGGTAACACACG		
Probe	(6FAM)CTCTTTGCAGGCATTTCTGACTGGGC(BHQ1)		

During ALV-J detection additional analytical sensitivity of the probe and primer system was evaluated by comparison of threshold RT-PCR cycle values (Ct) with those in a reference test system to detect ALV-J [8-10] (Table 2) using ALV-J DNA isolates extracted in a poultry farm in the Moscow Province. The results were similar to ones obtained with a Qin L test system [8] whose stated sensitivity was lower than 10 viral copies/sample; RT-PCR test based on conventional H5 and H7 primers was 100 times less sensitive to detect DNA of ALV-J [9, 10].

The 1× PCR buffer containing an exogenous virus-free chicken DNA was used as a negative control in all the tests. Negative controls did not induce any DNA amplification after 50 cycles of RT-PCR. We did not assess analytical sensitivity of a test system involving ALF, ALR, ALVPL probes and primers (see the Table 1) to detect all known ALV subgroups (including endogenous viruses). As a part of a multiplex RT-PCR, it can be applied as an inner amplification control.

Using the test system, we analyzed 1200 samples of chicken DNA collected in a poultry farm in the Moscow Province. ALV-J and ALV-K were detected in 42% and 8% of a chicken flock, respectively. The test did not reveal ALV-A and ALV-B in the tested sample. Moreover, we analyzed 97 DNA samples from chickens from the regions of Russia, i.e. Orenburg, Chelyabinsk, Kemerovo, Tyumen, Kaliningrad, Leningrad, Sverdlovsk, Novgorod regions and Krasnodar Territory. ALV-K was found in samples from the Kaliningrad (3 pcs), Leningrad (2 pcs), Sverdlovsk (5 pcs), and Novgorod regions (1 pc); ALV-A was detected in samples from the Leningrad region (7 pcs), and ALV-J was mentioned in the Sverdlovsk (3 pcs) and Leningrad regions (5 pcs). Since ALV-B was not detected, we can conclude that it is not common in Russia recently. Some positive samples were sequenced with primers mentioned in the Table 3. This verified specificity of the test system to detect ALV (A, J and K).

3. Primers for PCR and *gp85* gene fragment sequencing in various subgroups of avian leukosis virus (ALV)

Primers	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALVAF	GCCACACGGTTCCTCCTTAGA	443	ALV A
SEQA-KR	CGCGATCCCCACAAATGAGGAAA		

Table 3 continued

SEQJF	CCCTGGGAAGGTGAGCAAGAA	498	ALV J
SEQJR	CCTTTATAGCACACCGAACCGAA		
ALBF1	GGCCGAGGCCTCCCCGAAA	253	ALV B
SEQA-KR	CGCGATCCCCACAAATGAGGAAA		
ALVKF	CGGAGCATTGACAAGCTTTCAGA	466	ALV K
SEQA-KR	CGCGATCCCCACAAATGAGGAAA		

N o t e. Amplicons are fragments of *gp85* gene encoding GP85 virus coat protein.

A multiplex multilocus RT-PCR test system is intended for simultaneous detection of ALV-J and ALV-K to eradicate the virus. It was developed to eradicate ALV-J and ALV-K detected in lines of broilers from a poultry farm of the Moscow Province. As compared to other methods to detect and to eradicate avian leukosis virus, RT-PCR method is the most sensitive and advanced [8]. Total virus eradication is a quite challenging issue that requires a high-level testing programme. Vertical ALV transmission can be performed in absence of detected gs-antigen in chickens [1]. The infection control in some lines can be more complicated than in others. Presence of *ev21* locus containing an endogenous virus complicates ALV eradication because chickens become more susceptible to the infection [18]. A risk of ALV-J-associated viremia in broiler-type meat cross chickens can also restrict ALV eradication [1]. Extreme variability of the most common pathogenic ALV-J is a one of substantial difficulties associated with the virus spread control [1, 2]. The *gp85* gene of ALV-J coat protein isolated from different organs of the same body can demonstrate 94.9% variability of sequences [19]. A difference in amino acid sequences between the most distal isolates of the coat protein is 86.2% [20].

4. Primers and probes of the multiplex multilocus RT-PCR test system for simultaneous detection of avian leukosis virus (ALV) **J and K**

Primers	Nucleotide sequence (5′→3′)	Amplicon, bp	Subgroup
ALVKF	CGGAGCATTGACAAGCTTTCAGA	72	ALV K
ALVKR	GTGATTGCGGCGGAGGAGGA		(gene *gp85*)
KPL	(6FAM)CCACCTCGTGAG(dT-BHQ1)TGCGGCC-P		
JFF1F	GCCCTGGGAAGGTGAGCAAGA	139	ALV J локус 1
JJR	GGAAATAATAACCACGCACACGA		(gene *gp85*)
JJPLN	(ROX)CAGCAAGGGTG(dT-BHQ2)CTTCTCCG-P		
JNP	(ROX)TCCTCTCGA(dT-BHQ2)GGCAGCAAGGGTGTC-P		
JEF	CCTATTCAAGTTGCCTCTGTGGA	72	ALV J locus 2
JER	GCTTGCTCTATTTGGCCGTCAGA		(LTR)
JEP	(Cy5)CCATCCGAGC(dT-BHQ2)GCCTCCAGTCC-P		

To increase ALV-J detection reliability, we developed RT-PCR test system equipped with an additional JJPLN probe for *gp85* gene encoding the virus coat protein, and JEP probe intended for a long terminal repeat (LTR) fragment in ALV-J genome (Table 4). The test system increased ALV-J detectability by 2.3% as compared with one mentioned above.

A strategy of multilocus RT-PCR equipped with additional probes is also suitable for detection of retroviruses, coronaviruses and other microorganisms characterized by substantially variable genome. At the first stage of the programme, we used cloaca swabs collected in 1-day chickens to detect ALV-J and ALV-K. Further, we used feather pulp containing significantly greater virus amount than plasma and other tissues as a biomaterial. In a point of fact, ALV persists in feather pulp longer than in plasma [21]. As distinct from standard DNA isolation in blood, DNA isolation from feather pulp is a non-invasive labor saving method resulting in greater amount of DNA whose preparation does not contain PCR inhibitors. Aseptic blood sampling requires sterile tubes and needles for each chicken. In turn, only gloves and microcentrifuge tubes are used for a feather test [22]. While testing poultry for Marek's virus and ALV-J,

DNA isolation in feather pulp demonstrated better PCR findings than splenic DNA isolation [23].

After ALV penetration into a cell a reverse transcriptase and two copies of a single-stranded retroviral genome are released from a capsid. Then, a double-stranded DNA intermediate is formed that can built in a host's cell genome in presence of integrase [24]. Similarly to other types of different stages of retroviral development, the virus can be detected by RT-PCR [25. That is why, it would be quite invidiously to draw a conclusion about provirus detection only if a reverse transcription is not carried out. As for avian leukosis virus, detection of nucleic acids rather than viral antigens enables rejection of poultry with temporarily inactive provirus decreasing virus persistence in a flock. Stress is one of factors leading to reactivation of the infection [26]. We compared analytical sensitivity of the RT-PCR test systems with/without a reverse transcription. A reverse transcription resulted in up to 2 orders of magnitude greater increase in ALV-J and ALV-K detection sensitivity in tissues with active infection. This provides several additional opportunities to improve analytical sensitivity of ALV detection.

Using the multiplex multilocus RT-PCR test system, we carried out 7 cycles of screening of 4 initial and 6 experimental lines of boiler-type meat cross chickens (initial number — 9029). In the beginning of the ALV-J and ALV-K eradication program chickens with neoplasia were 17-26% depending on the line (the peak value was observed in a line with *ev21* locus). On day 265 after the start of the program, only three chickens of 2621 birds (0.10%) demonstrated neoplasia. Total percentage of samples containing ALV-J and ALV-K DNA among 2621 individuals at the age of 265 days was 0.67 % and 0.04 %, respectively.

Thus, our multiplex multilocus RT-PCR test systems intended for simultaneous detection of the most common ALV subgroups (A, B, J and K) and simultaneous detection of ALV-J and ALV-K demonstrated highly efficient detection and eradication of ALV.

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