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Original scientific article

DIAGNOSIS OF AVIAN LEUKOSIS VIRUS SUBGROUP J IN ASYMPTOMATIC COMMERCIAL LAYERS USING qPCR

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Avian Leukosis Virus (ALV), one of the dangerous retroviruses threatens the poultry industry, is oncogenic and can easily transmit. Over the past three decades, the poultry industry has been significantly affected by subgroup J (ALV-J) in particular, known for its ability to spread both horizontally and vertically. The fact that a commercial vaccine has not yet been developed against ALV increases the risk potential. For these reasons, positive cases should be identified to control ALV infections and minimize infection. A prompt and accurate diagnosis is vital to detecting infected birds. For this purpose, sampling was performed in total 153 blood samples from 14 different asymptomatic commercial layer farm located in Afyonkarahisar, Türkiye. Herein, the presence, density and prevalence of ALV-J strain and whether it has an oncogenic effect was investigated by qPCR and western blot for the first time in Türkiye. The average contamination rate of ALV-J was calculated as 70.91% over all samples. However, p27 antigen that is the most abundant polypeptide encoded by the *gag* gene of ALV could not be detected. High prevalence of ALV-J suggests that ALV strains can be found asymptomatically without showing any symptoms.

Keywords: avian leukosis virus, laying hen, prevalence, qPCR, western blot

INTRODUCTION

Avian leukosis virus (ALV), a retrovirus, has been attributed to substantial commercial losses in the poultry industry since the early twentieth century (KOSLOVA *et al*., 2018) also responsible for various tumor diseases (QIN *et al*., 2013; KAYA, 2018), and the first known virus-

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related tumor cause (LI *et al*., 2021). ALV is categorized into seven subgroups (A, B, C, D, E, J, and K) according to the characteristics of viral coat glycoproteins (PAYNE and FADLY, 1997; WANG *et al*., 2012). Among these subgroups, J (ALV-J) stands out as the most prevalent, responsible for chicken tumors (PAYNE *et al*., 1997; LI *et al*., 2021) and inflicting more significant damage compared to other subgroups (GAO *et al*., 2012). ALV-J was initially identified in meat-type chickens and named subgroup J due to its unique envelope characteristics (PAYNE *et al*., 1991). Since its discovery, ALV-J infection has been extensively documented across various countries in the Americas, Asia, and Oceania (BAI *et al*., 1995; SUN and CUI, 2007).

ALV has been identified as a significant co-factor in poultry diseases (ISFORT *et al*., 1994). The exacerbation of these diseases due to concurrent ALV infection is likely attributable to immunosuppression. Consequently, prompt identification and removal of virus-shedding birds are imperative for controlling and reducing ALV infections (QIN *et al*., 2013). However, in recent years, various tumors, including hemangiomas induced by ALV-J, have surfaced in breeder and commercial chicken flocks (GAO *et al*., 2010; JUSTICE *et al*., 2015). ALV-J has the strongest pathogenicity and infectivity among all subgroups, and it induces the most clinical cases in chicken, estimated to account for more than 90% of avian leukosis (LI *et al*., 2019). ALV-J can transmit easily both vertically and horizontally and there is no commercial vaccine available (QIN *et al*., 2013; CHEN *et al*., 2018; SWAYNE, 2020). A prompt and accurate diagnosis is vital to prevent a possible ALV outbreak. Blood, plasma, meconium, cloacal and vaginal samples, egg cells, embryos, and tumors are among the most frequently utilized samples for detecting ALV (QIN *et al*., 2013). The primary method for directly diagnosing ALV infection typically involves testing for the ALV gs antigen, a protein encoded by the *gag* gene of ALV, rather than relying on antibody assays. p27 is the most abundant among the structural polypeptides shared by all members of the endogenous and exogenous ALVs group (FADLY, 2000). However, distinguishing between endogenous and exogenous ALVs using direct assays based on the detection of p27, which is shared by both groups of viruses, is not feasible (PAYNE *et al*., 1993). It has also been reported that antigen tests, in particular, cannot be used reliably for ALV screening in the poultry industry (SMITH *et al*., 1998). However, quantitative PCR (qPCR) method, which has recently been used in the identification and quantification of ALV, has been accepted as subgroup-specific and much more sensitive, rapid and useful than traditional antigen tests (KIM *et al*., 2002; QIN *et al*., 2013; DAI *et al*., 2015; CHEN *et al*., 2018).

The study was conducted to assess the status of ALV infection in Türkiye, which has an important place in the world poultry sector, in terms of ALV. Afyonkarahisar, where commercial poultry is common, was chosen as the pilot region. Diagnosis of ALV-J has been investigated by qPCR method using two specific pairs of primers. In addition, p27 antigen was analyzed by western blot in individuals whether they were qPCR positive.

MATERIALS AND METHODS

Approval for this study was granted by the Akdeniz University Animal Experiments Local Ethics Committee (Approval no: 2018/48).

Sampling

A total of 153 chicken's (*Gallus gallus domesticus*) blood samples, aged from 5 to 7 weeks old, were randomly collected from 14 different laying hen commercial farms which were in the Afyonkarahisar region. Approximately 3-4 cm^3 of blood sample was taken from the vena brachialis of these asymptomatic chickens by using sterile cannula and transferred to pre-numbered sterile tubes containing EDTA anticoagulant.

RNA extraction and cDNA synthesis

Individual total RNAs were extracted from blood samples using the ExiPrep Plus Viral DNA/RNA Kit (Bioneer, Daejeon, South Korea). Then, the purity level of the RNAs obtained by using micro-volume spectrophotometer (Denovix, Wilmington, Delaware, USA), its suitability and whether its concentrations are sufficient have been checked. The RNA samples were preserved at -80°C. Two micrograms of extracted total RNAs underwent conversion to cDNA through reverse transcription using the AccuPower RT-PCR PreMix Kit (Bioneer, Daejeon, South Korea) in a Thermal Cycler.

qPCR amplifications

The qPCR amplifications were conducted on a LightCycler 96 Real-Time PCR instrument utilizing the SYBR Green fluorescent dye method, following the manufacturer's instructions, and employing the AccuPower 2x GreenStar qPCR Master Mix Kit (Bioneer, Daejeon, South Korea). Reactions contained 10 µl of 2X GreenStar Master Mix, 5 µl/100 ng of template DNA, 1 µl/10 pmol of each primer (forward and reverse) and 3 µl of PCR grade water in a final volume 20 µl. Two different primer sets reported in the literature were used to detect the presence of the ALV-J strain. Two different primer sets confirmed each other and ensured the reliability of the results (Table 1). The qPCR protocol consisted of an initial pre-denaturation cycle at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 25 seconds. A blank reaction mix was used as negative control and checked positive samples using qPCR to confirm the quantification a second time.

Table 1. Primer sets used to diagnose of ALV-J

Primer	Primer Sequence	Product size	Reference
ALV-J 1	F: TGTGTGCGTGGTTATTATTTC R: AATGGCGAGGTCGCTGACTGC	144 bp	(DAI *et al*., 2015)
ALV-J 2	F: GGATGAGGTGACTAAGAAAG R: CGAACCAAAGGTAACACACG	545 bp	(SMITH *et al*., 1998)

Protein purification and western blotting

Total proteins were purified from blood samples of each individual by using centrifugation method at 2500 g for 5 min. Protein quantification in the pellet part of the samples was made using spectrophotometer. After loading dye (Sigma–Aldrich, Saint Louis, Missouri, USA) is added onto the protein from each sample, lysates were separated using 4-12% NuPAGE Bis-tris gels (Invitrogen, Carlsbad, California, USA) and the separated lysates were transferred to nitrocellulose membranes (Millipore, Billerica, Massachusetts, USA). Following, blocking 1 h

with 5% non-fat milk powder at room temperature and washed with Tris Buffered saline Tween (TBST). The membranes were incubated with mouse anti- p27 antibody (MybioSource, San Diego, California, USA), mouse anti-β-actin (Sigma–aldrich, Saint Louis, Missouri, USA). β-actin antibody was used as a loading control. Then washing with TBST, membranes were treated with rabbit and mouse IgG ECL secondary antibody and HRP-conjugated secondary antibody (GE Healthcare, Chicago, Illinois, USA) for 1 h. After washing with TBST, the blots were visualized using enhanced chemiluminescence (ECL) substrate kit (Thermo Scientific, Waltham, Massachusetts, USA). Images were captured and the band density was analyzed with GeneTools software (Syngene, Cambridge, UK).

Statistical analysis

The prevalence of infection was determined by calculating the percentage of infected individuals divided by the total number of individuals sampled. Raw data of RT-qPCR were processed by LinReg software (RAMAKERS *et al*., 2003) to calculate Ct verification and PCR efficiency. A sample was considered positive when the Ct value was below that of the negative control (sterile water). Obtained Ct values were used for expression confirmation analyses by Microsoft Excel-based geNorm 3.5 software (http://genomebiology.com/2009/10/6/R64). The differences in the number of infected individuals and the average Ct values of the enterprises with positive results in terms of ALV were investigated by ANOM test in Minitab 21 (Minitab Inc, State College, Pennsylvania, USA).

RESULTS

qPCR results

The presence of ALV-J was detected in different numbers of individuals in all 14 laying hens farm where samples were collected (Figure 1). According to the results of the two primer sets used in qPCR analysis, 111 and 106 individuals from 153 blood samples were found positive for ALV-J, respectively. ALV-J prevalences in terms of primer 1 (DAI *et al*., 2015) and primer 2 (SMITH *et al*., 1998) were determined as 72.54% and 69.28%, respectively.

The ANOM analysis revealed that the disparity in Ct (cycle threshold) values obtained from qPCR among enterprises was not statistically significant. Unlike others, only one farm differs slightly in terms of ALV-J prevalence (Figure 2).

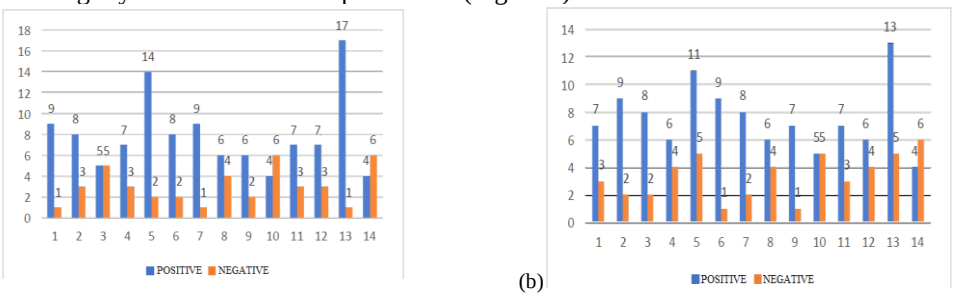

Figure 1. Quantification of ALV-J positive and negative individuals using qPCR with primer-1 (DAI *et al*., 2015) and primer-2 (SMITH *et al*., 1998)

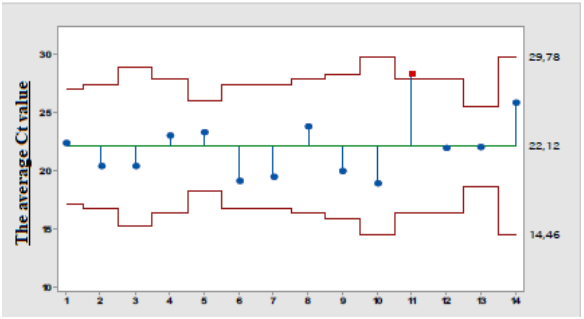

Figure 2. Comparison of Ct values determined for farms according to ALV-J primer with One-Way ANOM test

Western blot

No bands were obtained in the membrane with the p27 antigen. These results indicated that there are no signs of a disease or cancer caused by ALV-J in chickens that are found to be contaminated in terms of ALV-J strains.

DISCUSSION

In this study, a total of 153 samples from 14 distinct farms underwent screening with ALV-J-specific primer sets using a qPCR device. Subsequently, the samples were tested for the presence of p27 antigen using the western blot method. As a result of qPCR, it was determined that ALV-J strain was present in all farms and the average prevalence was calculated as 70.91%. However, p27 antigen was not found in the positive individuals. In the positive individuals, the absence of antigen and the absence of any lesions suggest that it may be associated with asymptomatic infection of ALV-J. In fact, the absence of p27 antigen in serum can be interpreted in two different ways; 1- asymptomatic infection of ALV-J, and unexpressed antigen, 2- there may be an infection with a low viral load and therefore the antigen test may be negative, but the qPCR test may be positive. The first hypothesis is more plausible, as animals have no signs of lesions or disease, and viral loads are high. On the other hand, it would not be correct to establish a connection with the presence of antigen and the time of blood sampling. Studies have indicated that ALV-J can be detected in the blood and cloaca as early as one week after infection (QIN *et al*., 2013).

Although the first discovery of ALV-J was in meat-type chickens in 1988, no cases of ALV-J tumors in laying hens were observed at any site worldwide until 2004. However, outbreaks of ALV have been reported in laying hen flocks in China (GAO *et al*., 2012). As a result of diagnostic studies on ALV-J, the importance of ALV-J infections in terms of bird health has been understood in the last thirty years (DENG *et al*., 2021). ALV-J remains a significant concern in China, as it can induce neoplastic diseases in poultry (XIANG *et al*., 2022). The presence of multiple sources of ALV infection underscores the importance of identifying and addressing each source diligently when formulating control plans (ZHANG *et al*., 2020). The

mortality rate in chicken flocks reached 15% In the ALV-J epidemic that started in six provinces of China in 2018 (ZHOU *et al*., 2018).

A few of studies had been done earlier by using antibodies not qPCR in Türkiye on the presence and prevalence of ALV-J. SEN *et al*. (2000) found ALV p27 antigens between 10% and 42% in 10 farms in Türkiye, including 3 commercial layers and 7 broilers. Similarly, KONAK (2009) investigated that the presence of ALV p27 antigen by ELISA method. As a material, 585 egg samples collected from 6 different districts were used to represent Aydın region. ALV p27 antigen was detected in 110 (72.85%) of 151 flocks and 279 (47.69%) of 585 eggs. In another study, totally 87 broilers (70 chicks and 17 chickens) from Marmara region investigated that the presence of antibodies to ALV-J (TURAN and YILMAZ, 2005). They reported that antibodies to ALV-J were detected in 13 (76%) of the17 broiler breeders. The results of a few numbers of studies agree with the results of this study. As a result of these studies, it is understood that ALV has been present and widespread in Türkiye for a long time.

In the present study, the presence and intensive of ALV was investigated for the first time in Türkiye by using qPCR method. Also, chickens were obtained from commercial farms belonging to the Afyonkarahisar region as the project material, in such a study for the first time. The positive results with both primers showed that ALV-J strains were present and widespread in that region. The results of this study concern not only Afyonkarahisar region but the whole poultry sector. The absence of an oncogenic finding was considered as a promising for poultry industry in the investigated region of Türkiye. Nevertheless, flocks should be kept under control against ALV. It is thought that, with new studies to be carried out on this subject, and we should paymore attention to the diseases caused by ALV.

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DIJAGNOSTIKA PODGRUPE J VIRUSA PTIČJE LEUKOZE U ASIMPTOMATSKIM KOMERCIJALNIM SLOJEVIMA U TURSKOJ KORIŠĆENJEM qPCR

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Izvod

Virus ptičje leukoze (ALV), jedan od opasnih retrovirusa koji ugrožava industriju živine, onkogen je i lako se prenosi. Posebno podgrupa J (ALV-J), koja se može prenositi horizontalno i vertikalno, nanosi značajnu štetu živinarskoj industriji poslednjih trideset godina. Činjenica da komercijalna vakcina još nije razvijena protiv ALV povećava potencijal rizika. Iz ovih razloga treba identifikovati pozitivne slučajeve kako bi se kontrolisale ALV infekcije i minimizirala infekcija. Brza i tačna dijagnoza je od vitalnog značaja za otkrivanje zaraženih ptica. U tu svrhu, uzeto je ukupno 153 uzorka krvi sa 14 različitih asimptomatskih farmi komercijalnih slojeva lociranih u Afjonkarahisaru, Turska. U radu su prisustvo, gustina i prevalencija ALV-J soja i da li ima onkogeni efekat ispitani qPCR i vestern blotom, po prvi put u Turskoj. Prosečna stopa kontaminacije ALV-J je bila 70,91% za sve uzorke. Međutim, antigen p27 koji je najzastupljeniji polipeptid kodiran gag genom ALV nije mogao biti otkriven. Visoka prevalencija ALV-J sugeriše da se sojevi ALV mogu naći asimptomatski bez ikakvih simptoma i da mogu biti uobičajeni u Turskoj.

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