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Genetic characterization of Avian leukosis virus subgroup J from layer and broiler breeder chickens in Egypt

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ABSTRACT

Avian Leukosis Virus subgroup J (ALV-J) is an oncogenic virus infecting meat-type and layer chickens causing severe economic losses. This study aimed to detect ALV-J in suspected chicken flocks in Egypt during the period of 2018- 2022 in addition to genetic analysis of the detected viruses. Fifty seven samples were collected from suspected chicken flocks. Samples were examined by polymerase chain reaction (PCR) for different ALV subgroups in addition to histopathological examination. Eight samples from local breeds were positive for ALV-J; 4 from layer chicken and 4 from broiler breeder chicken. All samples were negative for other ALV subtypes. Gene sequencing was done for all positive samples for gp85 gene; all of them were genetically related to each other with homology percent ranged between 91-97% on amino acids level, Also they showed amino acids mutations ranged between 4 to 14 in comparison with HPRS103/EgM/00 with detection of new mutations like F41 deletion in sample 6 and 117N insersion in sample 1 in addition to many substitutions that not recorded previously in Egyptian strains. Histopathological investigation of positive flocks showed lymphocytic and myelocytic cells infiltration of liver, spleen and kidney. Additional studies are required to assess the effect of these new mutations on pathogenicity of ALV-J strains.

INTRODUCTION

ALV belongs to genus Alpha-retrovirus of the fam-ily Retroviridae **(Payne et al. 1991)** and was classified based on envelope properties, host range, and cross-neutralization into 11 viral subgroups. Subgroups A to D, J and K were considered exogenous viruses, while subgroups E to I were classified as endogenous viruses **(McNally et al. 2010; Bande et al. 2016; Zhao et al. 2018)**.

The first detection of new group of ALV-J was at 1980 in the UK **(Payne et al. 1991 and**

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1992). ALV can be transmitted in chickens horizontally and vertically and prevention of vertical infection can be done by eradication of infected chickens **(Chai and Bates, 2006; Chesters, et al. 2006).** ALV-J can cause different tumors mainly in broiler breeders more than other ALV subgroups **(Qu, et al. 2016),** in addition to many studies reported ALV-J infection in layer chickens **(Xu et al. 2004; Gao et al. 2012)**.

ALV-J infection in different breeds of chicken was reported in European countries, United States, and China causing serious economic losses **(Fadly and Smith, 1999; Sun and Cue, 2007; Meng et al. 2018)**. ALV-J was detected in broiler breeders, Layers, broiler chickens and ducks in Egypt **(Mousa and Abdel-Wahab, 2009; Kilany et al. 2015; Yehia et al. 2020; Soliman et al. 2023; Fotouh et al. 2024).**

The ALV-J infection is usually accompanied with tumor development in internal organs, immune suppression, decreased weight gain, decreased egg production, and increased mortality rates in addition to occurrence of both lymphoid and myeloid leukosis in chicken **(Fadly and Payne, 2002; Li et al. 2017; Yehia et al. 2020)**. Until now, there is no treatment or vaccination available for ALV-J infection. Therefore, Establishment of biosecurity measures with rapid and accurate diagnosis of ALV-J infections are very important for effective eradication of infected hosts and prevention of serious economic losses.

The ALV genome is a positive sense single strand RNA of about 7200 bp and consists of three open reading frames (gag, pol, and env) that can be translated to the specific glycoprotein. The env gene consists of the gp85 and gp37 regions. The gp85 gene is about 921 bp in length and exhibits genetic variations compared to other genes. Also, it is the most important gene as it the major subgrouping determinant and will be translated into viral proteins that involved in viral infection, pathogenicity and tumor formation **(Payne and Venugopal, 2000; Holmen and Federspiel, 2000; Liu et al. 2021)**. Moreover, the gp85 gene evolves quickly in ALV-J compared with other ALV subgroups when under host immune pressures. Thus, it is important to monitor the evolu-tion of this gene for detection of new mutations that may affect the pathogenicity of ALV **(Pan et al. 2012; Yehia et al. 2020)**.

Although virus isolation and histopathological examination of tumors have been routinely used for identification of avian oncogenic viral infections, these methods are labor-intensive and time-consuming. In addition to virus isolation is difficult in case of multiple infections are present. Tissue histopathology alone is often difficult to diagnose the cause of tumors that are influenced by different oncogenic viruses. These lesions include lymphocytic and myelocytic infiltration in liver, spleen and proventriculus with mitotic figures **(Davidson, 2001; Wen et al. 2018)**. Molecular diagnosis by PCR is currently the most precise method for differential diagnosis of oncogenic viral infections as it is more sensitive and specific technique **(Davidson, 2009)**.

This study aims to identify ALV in flocks of layer and broiler breeder chickens in Egypt during the period of 2018- 2022 using PCR, histopathology and studying the genetic evolution within the detected viruses.

MATERIALS and METHODS

Sampling

During the period between January 2018 and December 2022, Samples were collected from 57 diseased chicken flocks (33 from layer flocks and 24 from broiler breeder flocks). These flocks were located in 10 Egyptian governorates; 17 from Sharkia, 11 from Qaliobia, 10 from Behira, 7 from Dakahlia, 4 from Kafr El Sheikh, 3 from Giza, 2 from Ismalia, 1 from Menofia, Gharbia and Damietta. The diseased chicken flocks showed mortality rates ranged between 10-20% and different clinical signs like emaciation, depression, decreased levels of

egg production, and enlargement of liver and spleen. Samples were collected from liver, spleen, and ovary from freshly dead chicken for PCR examination and histopathological examination.

Detection of ALV by PCR

The tissue samples were homogenized aseptically using sterile phosphate buffer saline in morters and pestles then centrifuged for 5 min at 5000 rpm, and the supernatant fluid was used for PCR examination. DNA isolation was performed using QiaAmp DNA minikit **(Qiagen, USA)** depending on the manufacturer's protocol.

Amplification of isolated DNA was done using Emeraldamp max PCR master mix **(Takara, Japan)** and specific primers for each ALV subgroups **(Table 1).** The PCR thermal profile began with initial DNA separation at 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing temperature according to each subgroup **(Table 1)** for 40 s and extension tem-perature at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR reactions were performed in simpli-Amp thermal cycler **(Applied biosystems, Germany)**.

The gene specific PCR products **(Table 1)** were separated by agarose gel electrophoresis using 1.5% agarose (Applichem, Germany). Gelpilot 100 bp plus ladder **(Qiagen, gmbh, Germany)** was used to determine the PCR product sizes. Finally, gel photos were captured using a gel documentation system **(Biometra, Germany)**

Table 1. Primer sequences, annealing temperatures, and PCR product lengths of ALV different subtypes.

Agent	Primer sequence	Annealing temperature	Product length	Reference
ALV-J	F: GGATGAGGTGACTAAGAAAG R: CGAACCAAAGGTAACACACG	60 °C	545 bp	Smith *et al.* 1998
ALV-A	F: GGATGAGGTGACTAAGAAAG R: AGAGAAAGAGGGGYGTCTAAGGAGA	55 °C	694 bp	Fenton *et al.* 2005
ALV-C	F: CGAGAGTGGCTCGCGAGATGG R: CCCATATACCTCCTTTTCCTCTG		1400 bp	Silva *et al.* 2007
ALV-B or D	F: CGAGAGTGGCTCGCGAGATGG R: AGCCGGACTATCGTATGGGGTAA	60 °C	1100 bp	

DNA sequencing of the partial envelop gene and phylogenetic analysis

Positive ALV-J samples were subjected to molecular characteriza-tion of gp85 region of envelope gene. QIAquick Gel Purification Kit **(Qiagen, Germany)** was used for purification of positive PCR products. DNA sequencing was done using BigDye Terminator v3.1 Cycle sequencing Kit **(Applied Biosystems, USA)** and specific primers for ALV-J. The DNA sequences were recognized using ABI 3500 Genetic Analyzer **(Life Technologies, USA)**. The obtained sequences were uploaded and published in the National Center for Biotechnology Information (NCBI).

The amino acid sequences in this study were aligned with other ALV-J strains obtained from NCBI database including HPRS103/EgM/00 strain which was used as a reference strain, and other strains that were previously detected from Egypt, USA, and China during the period of 2000-2024 using the Clustal W program of Bioedit software version 2.3 **(Hall, 1999)**. Identity percentages between nucleotides and amino acids sequences in the current study and other published sequences in the NCBI database were done using same software**.** Also, phylogenetic tree was constructed for these sequences using

MEGA version 6 (www.megasoftware.net) by neighbor joining tree model with 1000 bootstrap replicates **(Tamura et al. 2013)**.

Histopathological examination

Liver, spleen, kidney, intestine and ovary samples of positive ALV-J farms were collected in 10% buffered formalin, The fixed tissues were embedded in paraffin, sectioned at 4 µm thick and stained with haematoxylin and eosin as described in **(Bancroft and Layton, 2013)** and the slides were examined under light microscopy.

RESULTS

Molecular identification by PCR

Eight samples were positive for ALV-J out of fifty seven samples **(Table 2)** and all samples were negative for other ALV subtypes.

Table 2. Epidemiological data of ALV-J positive chicken flocks

Sample ID	Age	Year	Host	Breed	Governorate	Virus name
1	360 days	2018	Layer chicken	Baladi	Qaliobia	Egy1/ALV-J
2	160 days	2018	Layer chicken	Baladi	Sharkia	Egy2/ALV-J
3	138 days	2018	Layer chicken	dokki 4	Sharkia	Egy3/ALV-J
4	250 days	2019	Layer chicken	Baladi	Qaliobia	Egy4/ALV-J
5	180 days	2021	Broiler breeder chicken	Baladi	Qaliobia	Egy5/ALV-J
6	350 days	2021	Broiler breeder chicken	Saso	Qaliobia	Egy6/ALV-J
7	80 days	2021	Broiler breeder chicken	Baladi	Sharkia	Egy7/ALV-J
8	270 days	2022	Broiler breeder chicken	Baladi	Sharkia	Egy8/ALV-J

Genetic analysis of the gp85 region of ALV-J envelop gene.

Eight PCR positive ALV-J samples representing eight poultry farms were selected for partial gp85 gene sequencing and their sequences were uploaded to NCBI database with accession numbers PQ241043, PQ241044, PQ241045, PQ241046, PQ356997, PQ356998, PQ346901, and PQ346902. They were aligned and compared with Egyptian strain (HPRS103/EgM/00) on the amino acids level, where they showed amino acid mutations ranged from 4 to 14 **(Table 3)**. T46I were recorded in all strains, while some mutations were unique like 117N insertion in sample 1, T129A in sample 2, Y40H, Q44H, Q66R, and D124E in sample 3, R38K, Y40C, M45V, and H118P in sample 4, F41 deletion, G120R, and Q136R in sample6, and M45I and V115A in sample 7 (figure 1).

Table 3. Amino acids Mutations in ALV-J sequences.

Sample No.	Strain ID	Genebank Accession no.	Recorded mutations	No. of mutations
1	Egy1/ALV-J	PQ241043	T46I,V48A,V50I, I55V,117N insersion, H118Y, S125T, and T127A	8
2	Egy2/ALV-J	PQ241044	T46I, V48A,V50I, S125T, T127A, and T129A	6
3	Egy3/ALV-J	PQ241045	Y40H, Q44H, M45I, T46I, S54F, I55V, V56A, Q66R, H118Y, and D124E	10
4	Egy4/ALV-J	PQ241046	R38K, Y40C, F41S, M45V, T46I, V48A, V50I, S54F, I55V, V56A, H118P, S125T, T127A, and H140R	14
5	Egy5/ALV-J	PQ356997	F41S, T46I, V48A, V50I, I55V, V46A, H118Y, S125T, T127A, and H140R	10
6	Egy6/ALV-J	PQ356998	F41 deletion, T46I, V48T, V50I, I55V, V56A, H118Y, G120R, S125T, T127A, Q136R, and H140T	12
7	Egy7/ALV-J	PQ346901	M45I, T46I, V48A, V50I, I55V, V115A, H118Y, S125T, and H140T	9
8	Egy8/ALV-J	PQ346902	T46I , V48A, V50I, and I55V	4

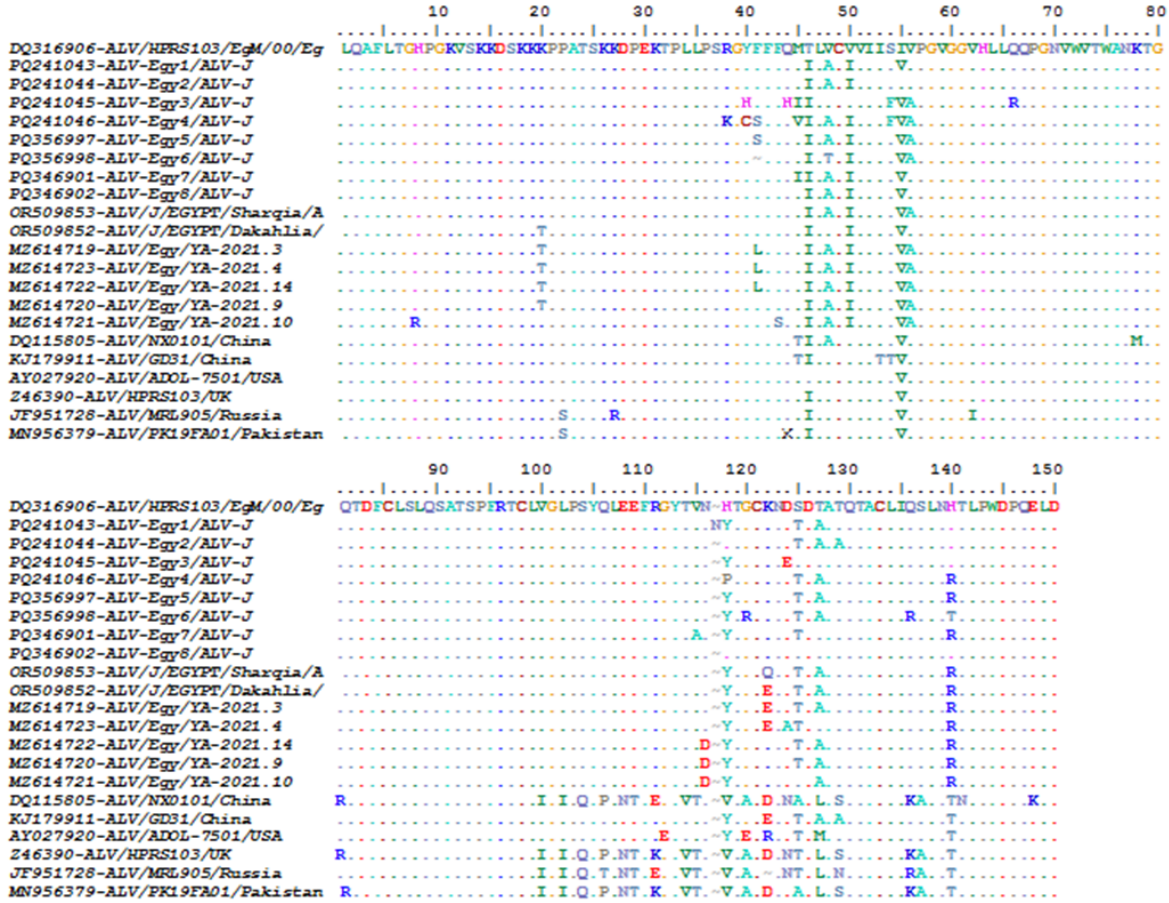

Figure1: Comparison of the gp85 gene sequences of the Eight ALV-J Egyptian strains in this study and other ALV-J strains. The top line refers to the amino acids numbers in the gp85 sequence of HPRS103/ EgM/00 strain. The letters refer to amino acid substitutions. The dots (.) refer to the same amino acids and dashes (−) refer to gaps produced in the alignment

The nucleotide identity% between ALV-J samples in the current study was 93%-99% and they showed high homology (92-99%) with previously sequenced Egyptian strains, ALV/ADOL7501/USA, and ALV/GD31/ China while they showed low homology (81% -84%) with ALV/NX0101/China, ALV/ MRL905/Russia, ALV/HPRS103/UK and AL-V/PK19FA01/Pakistan (figure 2).

The amino acid identity% between ALV-J samples in this study was 91%-97% and they showed high homology (89-99%) with other Egyptian strains, ALV/ADOL7501/USA, and ALV/GD31/China while they showed low homology (81%-86%) with ALV/NX0101/ China, ALV/MRL905/Russia, ALV/ HPRS103/UK and ALV/PK19FA01/Pakistan (figure 2).

Phylogenetic analysis of ALV-J strains revealed they are clustered together and with other Egyptian strains and related to strains like ALV/ADOL7501/USA and ALV/GD31/ China while they are away from other strains like ALV/MRL905/Russia, ALV/HPRS103/ UK ,ALV/PK19FA01/Pakistan, ALV/ WB11098/China and ALV/UD3/USA (figure 3).

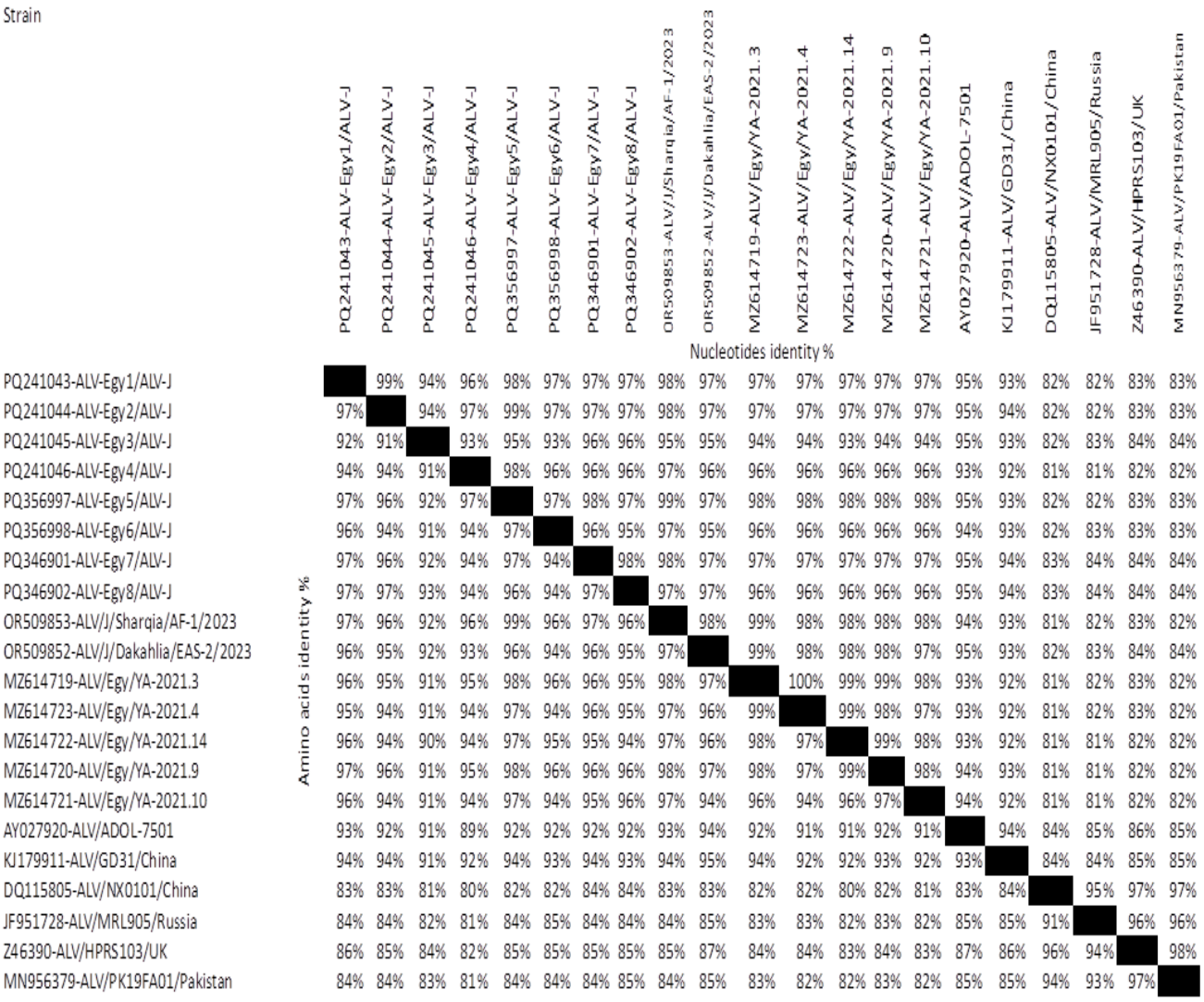

Figure 2:Nucleotide and amino acids identity percentages of ALV-J strains under study compared with other selected strains from Egypt, China, USA, Russia and Pakistan

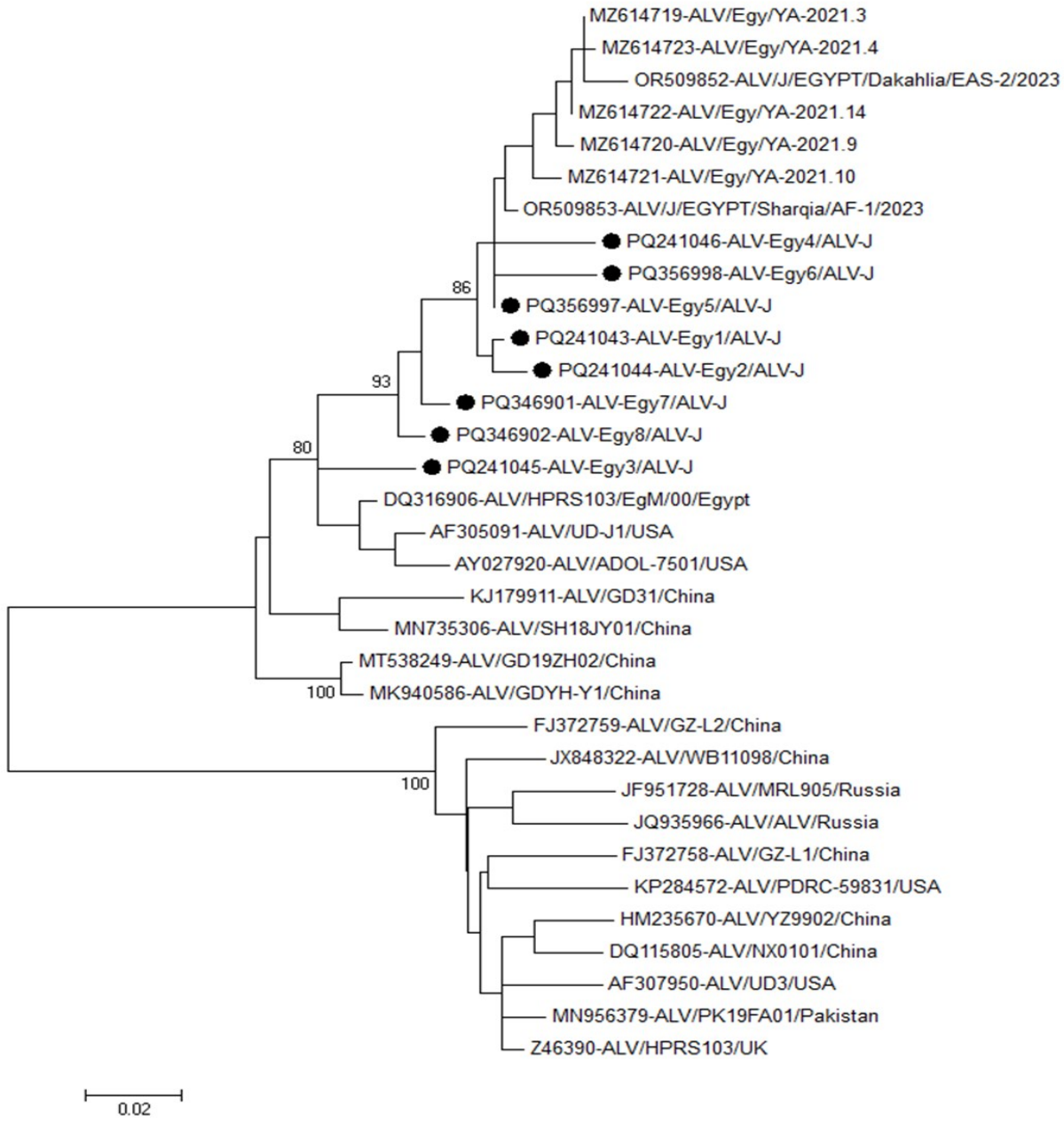

Figure 3: Phylogenetic tree of gp85 gene of ALV-J showing clusturing of Egyptian strains in the same group. The ALV-J strains in the current study were labeled with black circles

Histopathological findings

Microscopically, liver showed moderate hepatocellular necrosis with extensive lymphocytic cells infiltration. Spleen showed lymphocytic cells depletion and massive tumor lymphocytic cells infiltration associated with marked fibrous tissue proliferation. Ovary showed lymphocytic cells infiltration. Kidney showed severe tubular nephrosis and intertubular hemorrahges with obvious lymphocytic and myelocytic cells infiltration. Intestine showed lymphocytic cell infilteration of lamina propria associated with destruction of intestinal villi (figure 4).

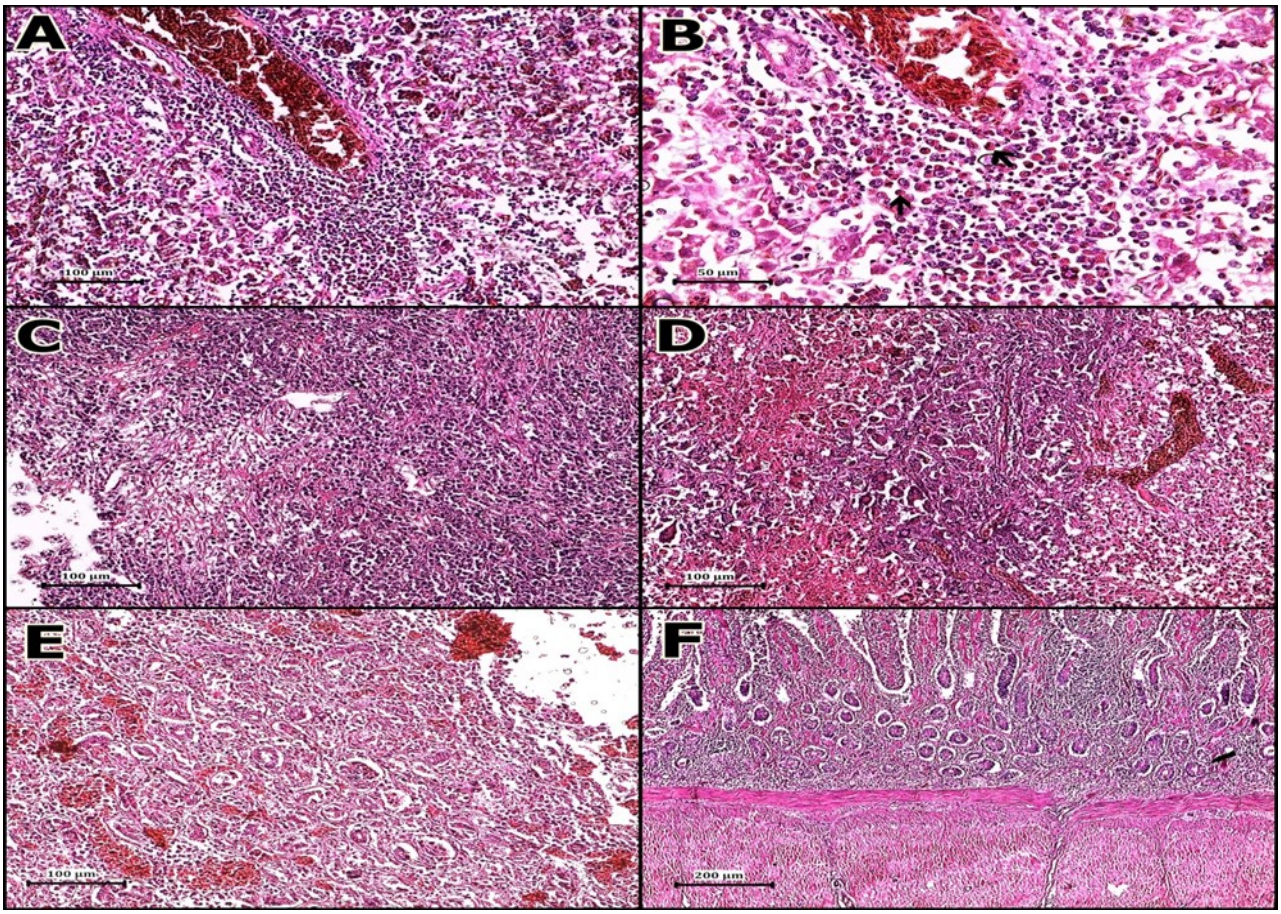

Figure 4: The histopathological examination of ALV-J infected broiler breeder chicken **(A): liver** showed marked hepatocytic degeneration and necrosis, congested central vein and massive lymphocytic cells within hepatocytic parenchyma. **(B): liver** with high magnification showed marked hyperplasia of myelocytic cells perivascular (arrow). **(C): spleen** showed diffuse lymphocytic depletion replaced by massive neoplastic lymphocytic cells and proliferation of fibrous connective tissue. **(D): ovary** showed severe destruction of ovarian tissue with congested blood vessels and lymphocytic cells infiltration. **(E): kidneys** showed severe tubular nephrosis and intertubular hemorrahges with obvious lymphocytic cells infiltration mixed with some myelocytic cells. **(F): intestine** showed marked enteritis, thickening of intestinal wall and destruction of intestinal villi which replaced by massive lymphocytic cells infiltration and hyperplasia of intestinal glands.

DISCUSSION

ALV-J is a serious tumor virus that can be transmitted vertically and horizontally and may contaminates poultry vaccines and induces economic losses in the poultry farms **(Mohamed et al. 2010; Li et al.,2017; Wang et al. 2020).** ALV-J Infection is difficult to control in absence of a vaccination program, thus the only available control method is eradication of infected commercial breeding flocks, so continuous surveillance is very important to detect ALV-J infections and to monitor genetic mutations that may affect virus pathogenicity and transmissibility **(Li, et al. 2025)**.

The ob-served clinical signs in ALV-J infections are depression, decrease in egg production, and retarded growth which are not a pathognomonic signs, so PCR and histopathological examination are important for confirmation of ALV-J infection **(Kilany et al. 2015; Eid et al. 2019).**

In the current study, ALV-J was detected in

eight chicken flocks out of fifty seven flocks (14%) by PCR; 4 layer and 4 broiler breeder. Recent studies reported similar results in Egypt **(Yehia et al. 2020; Soliman et al. 2023),** Also spleen and liver showed high tropism for ALV-J which agree with previous studies **(Fotouh et al. 2024).** All samples were negative for other ALV subtypes, Also other studies in Egypt didn't detect any ALV subtypes other than ALV-J by PCR **(Yehia et al. 2020; Soliman et al. 2023).**

In the current study 545bp DNA fragments of gp85 gene of eight positive samples were sequenced using the same PCR primers as this gene is highly mutated region that carry receptor binding site and play a critical role in viral pathogenicity and onchogenicity **(Meng et al. 2018; Ma et al. 2020).** The samples in the current study showed several mutations when compared with HPRS103/EgM/00 strain, the number of mutations ranged between 4 in sample no. 8 and 14 in sample no. 4 which indicate rapid evolution of ALV-J in Egypt in local breeds. Novel gp85 gene mutations were recorded in the current study like 117N insertion in sample 1and F41 deletion in sample 6 in addition to many substitution mutations. The amino acid mutations in gp85 gene of ALV-J occur duo to several passages of infections and immune pressure by host antibodies **(Wang and cui, 2006)** may cause variations in the pathogenicity, oncogenicity, and ALV-J host range **(Fotouh et al. 2024)**, and our findings act as an alarm for continuous genetic monitoring of ALV-J.

Phylogenetic analysis indicated that ALV-J strains were clustered in 2 major branches, the first branch include Egyptian strains and strains from USA like ADOL-7501and UD-J1and strains from China like GDYH-Y1 and GD31 while the second branch include HPRS103 strain from UK which consider prototype stain of ALV-J, most of China strains like NX0101 strain, strains from Russia and Pakistan. Also strains in this study showed 91-97% identity with each other in amino acid level and 81-86% identity with strains that belongs to second branch of phylogenetic tree. Our results agree with **Fotouh et al. (2024)** who found genetic differences in gp85 gene among isolated ALV-J strains from Egypt.

Histopathological examination revealed degenerative changes in addition to lymphocytic and myelocytic infilteration in differnt internal organs as liver, spleen and kidney. Similar results were reported previously in these organs from ALV-J infected chicken either experimentally infected chickens or field cases **(Wang et al. 2011; Yehia et al. 2020; Kheimar et al. 2021).**

Although several studies were conducted on controlling of ALV using antiviral agents like traditional Chinese herbal medicines and subunit vaccines and development of resistant breeds against ALV infection but until now none of these methods is applicable **(Sun et al. 2018; Wang et al. 2019; Koslova et al. 2020; Liu et al. 2022)**

Monitoring of breeder and layer flocks and imported chicks in addition to testing of attenuated vaccines for early detection of ALV are very important for early detection and elimination of positive cases to avoid economic losses.

CONCLUSION

AlV-J was detected by PCR in Egypt from eight chicken flocks during the period between 2018 and 2022. Nucleotide and deduced amino acid sequences of the gp85 gene of detected strains showed the presence of several mutations on the amino acid level and they varied from each other and from previously detected ALV-J strains from Egypt. Testing of local breeds as well as periodic molecular monitoring for the detected ALV-J strains with culling of positive flocks are recommended as there are no treatments or vaccines for ALV. Also, the full genome sequence and pathogenicity testing of detected strains are required to identify the pathogenicity of the recently detected ALV-J strains.

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