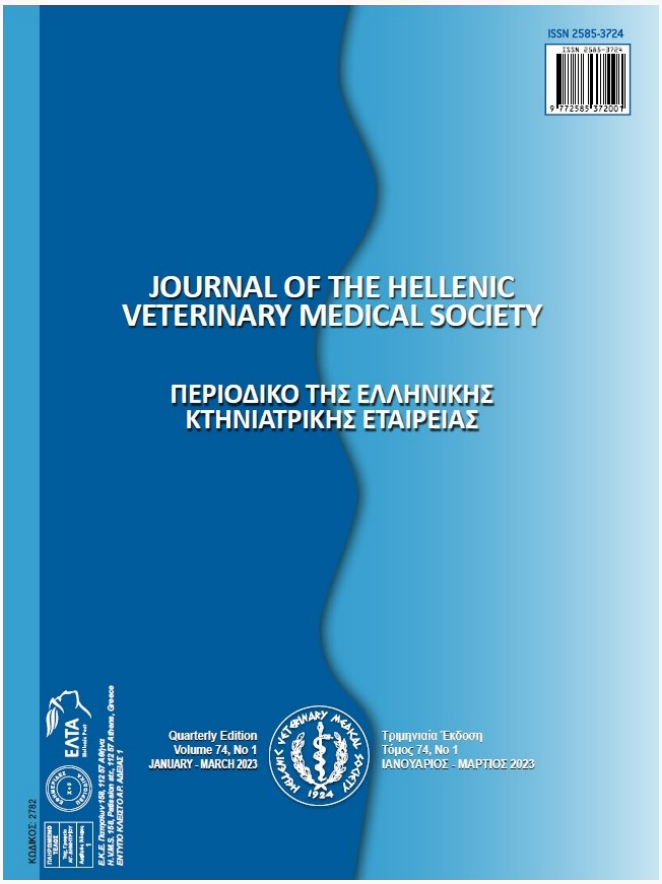
Journal of the Hellenic Veterinary Medical Society

Vol 74, No 1 (2023)



Viral analysis of tumor-bearing chicken flocks in Turkey over the last decade (2011-2020)

H Abayli, S Tonbak, R Ozbek, B Karabulut

doi: 10.12681/jhvms.28974

To cite this article:

Abayli, H., Tonbak, S., Ozbek, R., & Karabulut, B. (2023). Viral analysis of tumor-bearing chicken flocks in Turkey over the last decade (2011-2020). *Journal of the Hellenic Veterinary Medical Society*, *74*(1), 5267–5276. https://doi.org/10.12681/jhvms.28974 (Original work published April 11, 2023)

J HELLENIC VET MED SOC 2023, 74 (1): 5267-5276
ΠΕΚΕ 2023, 74 (1): 5267-5276

Research article
Ερευνητικό άρθρο

Viral analysis of tumor-bearing chicken flocks in Turkey over the last decade (2011-2020)

H. Abayli[1]*, S. Tonbak[1], R. Ozbek[1], B. Karabulut[2]

[1]*Department of Virology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey*

[2]*Department of Pathology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey*

ABSTRACT: Marek's disease virus (MDV), avian leukosis virus (ALV), and reticuloendotheliosis virus (REV) are important viral agents associated with neoplastic diseases that cause economic losses for the poultry industry worldwide. Immunosuppressive agents such as infectious bursal disease virus (IBDV), REV, and chicken anemia virus (CIAV) expose birds to other infections and reduce production performance. In this context, we aimed to identify and perform molecular analysis of the aforementioned viral infections (ALV, CIAV, IBDV, MDV, and REV) in the tissues of tumor-bearing chickens. Clinical samples were taken from 11 chicken flocks of different ages and races from some provinces (Adıyaman, Diyarbakır, Elazığ, and Malatya) in Turkey between 2011 and 2020. MDV positivity was determined in all 11 chicken flocks, while ALV sequences were determined in four of 11 MDV-1 positive chicken flocks. CIAV and ALV sequences were found together in two of 11 MDV-1 positive chicken flocks. The ALV subgroup J, IBDV, and REV were not detected in any chicken flocks. According to phylogenetic analysis, Turkish MDV strains obtained from this study were included in two different genogroups (genogroups I and II) with other MDV strains with pathotypes ranging from virulent (v) to very virulent plus (vv+). Co-infections of ALV and CIAV with MDV may worsen the current clinical situation. In this study, MDV was detected in two flocks vaccinated with Herpesvirus of turkeys. Other chickens were not vaccinated against any of the viral agents studied. Increasing vaccination and preventive measures against these viral pathogens in poultry in Turkey are recommended.

Keywords: chicken, tumour, Turkey, tissue, virus

Corresponding Author:
Abayli H., Department of Virology, Faculty of Veterinary Medicine, Firat University, 23110 Elazig, Turkey.
E-mail address: habayli@firat.edu.tr.

Date of initial submission: 18-12-2021
Date of acceptance: 10-07-2022

INTRODUCTION

Marek's disease virus (MDV), avian leukosis virus (ALV), and reticuloendotheliosis virus (REV) are among the most common naturally occurring viruses associated with neoplastic disease conditions in poultry (Payne, 2000). Infectious bursal disease virus (IBDV), REV, and chicken anemia virus (CIAV) are among the important immunosuppressive agents in poultry that cause production problems (Payne, 2000; Gimeno and Schat, 2018). These viruses are responsible for economic losses due to mortality and depressed performance (Payne, 2000).

Marek disease virus transforms T lymphocytes and forms skin and visceral tumors. In addition, the virus can cause immunosuppression, neurological symptoms, and ocular lesions until tumors become visible (Osterrieder et al., 2006). Marek's disease (MD) virus, or Gallid alphaherpesvirus 2 (GaHV-2), the causative agent of MD, is a herpesvirus belonging to the subfamily *Alphaherpesvirinae*, genus Mardivirus (Foster, 2018). Based on serological characteristics, MDV can be divided into three serotypes: MDV-1 (serotype 1, species Gallid herpesvirus 2), apathogenic MDV-2 (serotype 2, species Gallid herpesvirus 3), and an antigenically related Herpesvirus of turkeys (HVT or serotype 3, species Meleagrid herpesvirus 1). However, only MDV-1 can cause tumor formation in chickens (Zhang et al., 2012). All three MDV species are used in MD vaccines, alone or in combination. Based on the ability to break vaccine immunity, MDVs are divided into three pathotypes: v (virulent), vv (very virulent), and vv+ (very virulent plus) (Faiz et al., 2017). Among the more than 200 genes of the GaHV-2 genome, Marek's *Eco*RI-Q (MEQ) is one of the most extensively studied MDV genes. Serotype 1 MDV genome codes for a unique oncogene, MEQ, which is consistently expressed in all MDV latently infected or tumor cells. Thisgene encodes the oncoprotein (MEQ protein), a basic leucine zipper transcription factor composed of an N-terminal basic leucine zipper (**bZIP**) domain and a proline-rich C-terminal transactivation domain. Although the last 33 carboxy-terminal amino acids (aa) are required for transcriptional transactivation (Qian et al., 1995; Kung et al., 2001), the number of proline-rich repeats (PRR) at the transactivation site appears to be associated with the suppression of transcription (Chang et al., 2002).

ALV subgroups (A-E, J, and K) belong to the *Alpharetrovirus* genus (Shao et al., 2017). These subgroups are often associated with lymphoid leukosis, with tumors primarily in the bursa of Fabricius and visceral organs (Fadly and Venugopal, 2008), but ALV subgroup J (ALV-J) targets cells of the myeloid lineage, inducing late-onset myelocytomatosis (Venugopal et al., 2000). There are seven subgroups, A, B, C, D, E, J, and K, of ALVs identified in chickens according to the antigenicity of the viral envelope. They are also classified as being either exogenous or endogenous. Unlike the other subgroups of exogenous ALVs, subgroup E viruses are avian retrovirus-like elements that are transmitted genetically in a Mendelian fashion and are termed endogenous viruses (Shao et al., 2017; Fadly and Venugopal, 2008).

Reticuloendotheliosis virus (REV) is an oncogenic and immunosuppressive gammaretrovirus. The virus causes reticuloendotheliosis (RE), an avian disease mainly characterized by immunosuppression, runting–stunting syndrome, and chronic lymphomas (Niewiadomska and Gifford, 2013; Walker et al., 1983).

Chicken anemia virus (CIAV), genus *Gyrovirus* in the family *Anelloviridae* (Adams et al., 2016), has a worldwide distribution. It is recognized as a serious economic threat to broiler and specific pathogen-free (SPF) egg-producing industries (Balamurugan and Kataria, 2006). Infectious bursal disease (IBD) is a viral disease of young chickens that represents a persistent issue for poultry production worldwide. Some viral strains can induce high mortality (Samy et al., 2020). Immunosuppressive viral agents induce immunosuppression that facilitates infection by other avian pathogens and results in vaccination failures (Samy et al., 2020). Similarly, avian retroviruses can be found as contaminants in commercial vaccines and cause problems when their genome is integrated into the MDV and fowl pox virus genome (Fadly et al., 1996; Koyama et al., 1997).

In this context, we aimed to identify and perform molecular analysis of ALV, MDV, CIAV, IBDV, and REV infections in the tissues of chickens with tumors.

MATERIALS AND METHODS

Sample history and processing

Sampling dates were the decade from 2011 to 2020.In this study, clinical samples consisted of the tissues (heart, spleen, and livers) with diffuse whitish tumors foci of 11 chicken flocks with different production types in some provinces (Adıyaman, Diyarakır, Elazığ, Malatya) of Turkey. The history notes

of the affected chicken flocks included death and stagnation, weakness, and loss of appetite. The necropsy notes included increased size (2-3 times) of the liver and spleen and numerous white nodular structures with lesions of varying sizes (2 mm to 10 mm). Tissue specimens were fixated in the 10% formalin solution and placed in standard tissue processing cassettes. After routine tissue processing and paraffin embedding procedures, histological sections were taken from the paraffin blocks with a rotary microtome, stained with hematoxylin-eosin, and examined under routine light microscopy.Histopathological examination showed that the lesions were lymphomatous, consisting of neoplastic pleomorphic cells. Lymphoblasts and plasma cells were dominant in neoplastic foci. Lymphocytes of various sizes were also observed.

After microscopic examinations, non-paraffinized tissue samples such as spleen and liver were homogenized with 1X phosphate-buffered saline (PBS, pH 7.2–7.4), and the resulting homogenate was centrifuged at 1500 rpm.

DNA-RNA isolation from the supernatant was performed with the QIAamp MinElute Virus Spin kit (Hilden Germany) according to the manufacturer's instructions. The extracted nucleic acids were eluted in 50 μL elution buffer and kept at –20 °C until analysis.

Detection of avian oncogenic viruses (REV, ALV, and MDV)

To detect avian leukosis and reticuloendotheliosis from oncogenic viruses, we followed the polymerase chain reaction (PCR) procedure used by Ongor and Bulut (2011). Also, the p27 gene of ALV was amplified using PCR with gene-specific primers (Yunet al., 2013).

Specific primers of MEQ (Murata et al., 2013) and 132 repeats (Tian et al., 2011) were used to detect MDV,and reference PCR procedures were followed. A blank reaction consisting of primers but no DNA template was included to serve as a reagent control. The obtained amplicons were analyzed by electrophoresis (110V/40 min) in 1.5% (w/v) agarose-TAE (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) gel stained with ethidium bromide (EtBr).

Detection of other viruses (CIAV, IBDV)

Reference primers and PCR procedures were used to detect CIAV (Zhang et al., 2013) and IBDV (Sapats and Ignjatovic, 2000).

Sequence analysis

PCR products were run on gel electrophoresis, purified, and sequenced by a commercial sequencing service (Macrogen Europe, Amsterdam, The Netherlands). Sequencing was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems), using the BigDye Terminator v3.1 cycle sequencing kit (ApApplied Biosystems). The resulting bidirectional nucleotide sequences were aligned, edited, verified with BLASTN, and submitted to the GenBank database. Then, nucleotide sequences were aligned and compared with strains selected from GenBank using the Clustal W method. Sequence data have been submitted to the GenBank Nucleotide Sequence Database.

The nucleotide sequences for the MEQ gene of MDV were aligned with those of 35 MDV strains with different pathotypes belonging to different geographic regions selected from the GenBank database, using MEGA X (Kumar et al., 2018). Phylogenetic trees were generated using the 'maximum likelihood' method and the Tamura Nei model (Tamura and Nei, 1993) with 1000 bootstrap replicates. Also, alignment analysis of deduced MEQ complete amino acid sequences of the MDV strains in this study and other reference MDVs from China or the USA together with the vaccine strains was performed. Furthermore, the number of four consecutive prolines (PPPP) within the proline-rich repeats (PRR) of the transactivation domain, the proline content (%), and the presence of amino acid (aa) substitutions in the deduced aa sequence of MEQ genes were determined.

Similarly, the CIAV complete genome nucleotide sequences obtained in this study were aligned with 32 CIAV strains from different regions selected from the Genbank database, and a phylogenetic tree was constructed. Additionally, the amino acid sequences of CIAV (VP1, VP2, and VP3) were aligned with other strains, and a phylogenetic tree for the VP1 gene was constructed.

The homology of the sequences of the H2-H5 (ALV pol flanked sequences) and the p27 amplicons with the reference strains wereevaluated using BLASTN analysis.

Sequence Similarity and Identity Analysis

The similarity analysis between the MEQ gene of the MDV strains obtained from this study and those of 35 MDV strains/isolates with different pathotypes se-

lected from Genbank was calculated with SIAS software (http://imed.med.ucm.es/Tools/sias.html). The same method was used to calculate the similarity ratio between the complete genome nucleotide sequences of the CIAV strains obtained from this study and the others.

Recombination analysis

Two CIAV strain sequences from the current study and other sequences from GenBank were used to identify recombination events using Recombination Detection (RD), BootScan, GENECONV, Max-Chi, Chimaera, SiScan, and 3Seq methods implemented in the Recombination Detection Program (RDP)version 4.1 (Martin et al., 2015), with default settings. It was designed to accept the presence of recombination if recombination was identified with at least five of the seven methods (p-value <0.05).

RESULTS

PCR

In this study, MDV-1 was detected in all 11 chicken flocks with tumor foci in their organs.After PCR, 1063 nucleotides of MEQ and 314 nucleotides of 132 repeats were amplified. Two flocks were present with CIAV co-infections. For complete genomes of two CIAVs(CIAV/2011/Akcdg/TUR and CIAV/Dyrb-

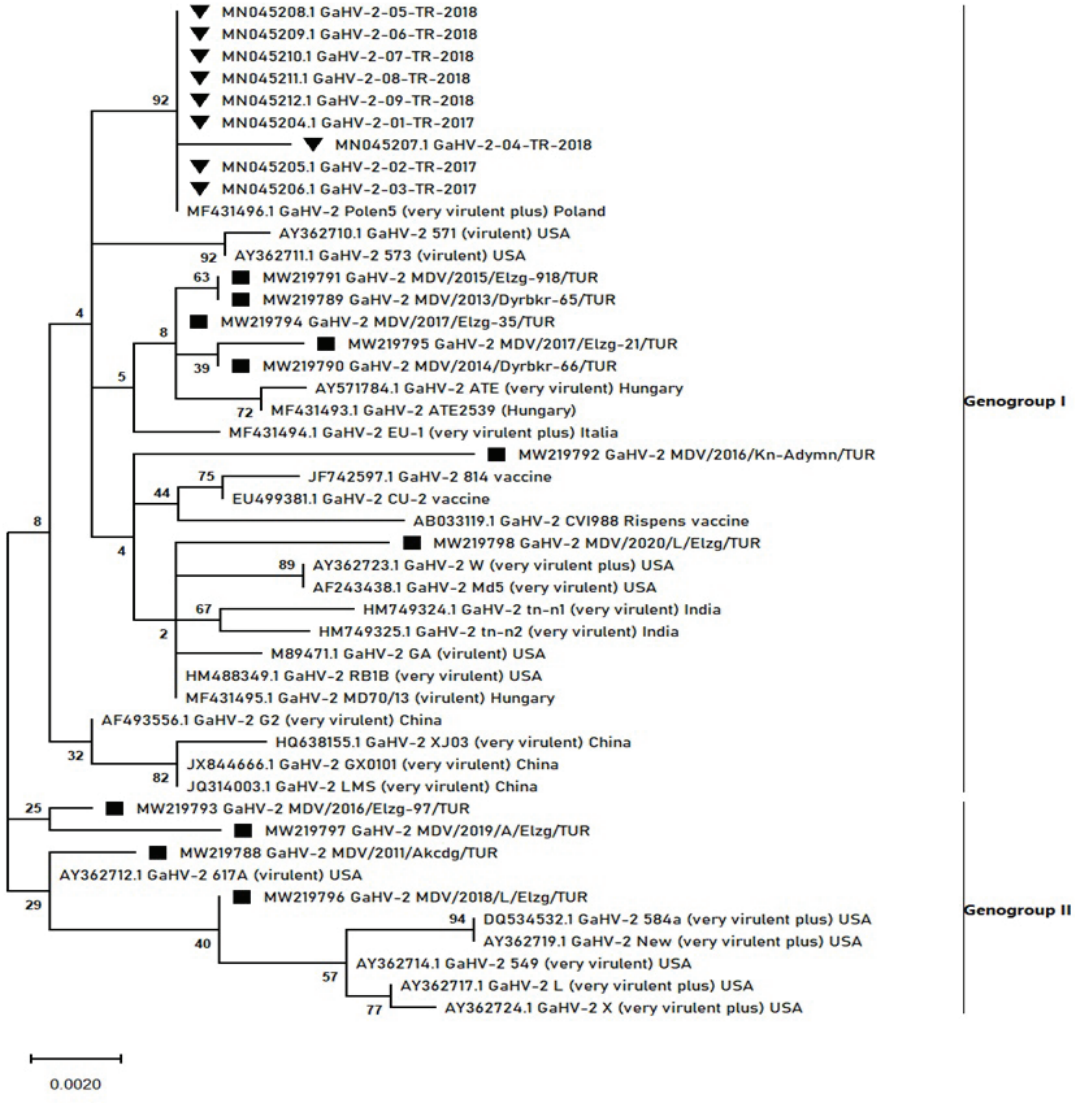


Figure 1 Phylogenetic analysis of the MEQ gene of MDVs. Phylogenetic trees were created using the Maximum Likelihood method (1000 replicates) and Tamura Nei model by the Mega X. Filled boxes represent the Turkish MDV strains obtained from this study, while the filled triangles represent other Turkish MDV strains.

kr-66/TUR) were amplified overlapping sequences of 1778 and 831 nucleotides length by PCR. As a result of PCR with primers H2-H5 and p27 for ALV detection, PCR products of 300-310 and 720 nucleotides in length were obtained in four chicken flocks, respectively.No amplification was found in the PCR with H5-H7 primers performed to detect ALV subgroup J. No amplicons were detected in any negative controls after PCR.

Sequence analysis

In this study, the MEQ gene of Turkish MDV strains encoded 339 amino acids, of which 71 to 73 (20.9-21.5%) consisted of proline. The number of PPPPs in the PRR of the transactivation domain of Turkish MDV strains varied from three to five. While the number of PPPPs was three in MDV/2011/Akcdg/TUR and MDV/2018/L/Elzg/TUR strains, this number was four in MDV/2016/Elzg-97/TUR and MDV/2019/A/Elzg/TUR strains. According to the phylogenetic tree results for the MEQ gene (Figure 1), Turkish MDV strains obtained from this study were included in two separate phylogroups (Genogroups I and II). According to this result, MDV/2011/Akcdg/TUR, MDV/2016/Elzg-97/TUR, MDV/2018/L/Elzg/TUR, and MDV/2019/A/Elzg/TUR strains were included in genogroup II, while others were included in genogroup I. Some changes in the MEQ amino acid sequences of Turkish MDV strains obtained from this study were as follows: E77K, Y80D, A88T, C119R, M126K, A139T, P153Q, Q169H, P176A, T180A, I200V, P217A, H240Y, A262T, and A294V. All the amino acid differences in the MEQ are shown in Table 1.

Table 1 MEQ amino acid alignment of Turkish and reference MDVs

	35	57	80	88	93	01	01	10	15	19	26	39	35	39	66	78	94	00	09	17	88	70	02	35	51	13	36	42	93	03	09
MW219789 MDV/2013/Dyrbkr-65/TUR	K	E	Y	A	Q	R	K	C	V	C	M	A	P	Q	P	T	P	I	L	P	P	C	H	A	E	C	G	A	F	A	L	I	P
MW219788 MDV/2011/Akcdg/TUR	.	.	D	.	.	.	.	.	.	R	.	T	Q	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
MW219790 MDV/2014/Dyrbkr-66/TUR	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
MW219791 MDV/2015/Elzg-918/TUR	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MW219792 MDV/2016/Kn-Adymn/TUR	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	T	.	.	.	.	V	.	.	.	.
MW219793 MDV/2016/Elzg-97/TUR	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
MW219794 MDV/2017/Elzg-35/TUR	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
MW219795 MDV/2017/Elzg-21/TUR	.	.	.	T	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
MW219796 MDV/2018/L/Elzg/TUR	.	K	.	.	.	.	.	.	.	R	.	T	.	.	A	A	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
MW219797 MDV/2019/A/Elzg/TUR	.	K	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
MW219798 MDV/2020/L/Elzg/TUR	.	K	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	T	.	.	.	.	.	.	.	.	.
MF431495.1 MD70/13 (v) Hungary	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
DQ534532.1 584a (vv+) USA	.	K	D	.	.	.	.	.	.	R	.	T	Q	.	A	.	.	.	.	A	.	.	.	T	.	.	.	V	.	.	T	.
AF243438.1 Md5 (vv) USA	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	V	.	.	T	.
AY362723.1 W (vv+) USA	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	V	.	.	T	.
HM488349.1 RB1B (vv) USA	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
MF431496.1 Polen5 (vv+) Poland	.	.	.	.	.	.	.	S	.	.	.	T	.	.	.	.	.	.	.	.	S	.	.	T	.	.	.	.	.	.	.	.	.
MF431494.1 EU-1 (vv+) Italia	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	R	.	.	.	.	.	.	.
JX844666.1 GX0101 (vv) China	.	.	.	.	.	.	.	.	A	.	.	.	.	.	R	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
JQ314003.1 LMS (vv China	.	.	.	.	.	.	.	.	A	.	.	.	.	.	R	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
M89471.1 GA (v) USA	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
MF431493.1 ATE2539 (Hungary)	.	.	.	T	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
AY362712.1 617A (v) USA	.	.	.	.	.	.	.	.	.	R	.	T	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
AY362717.1 L (vv+) USA	.	K	D	.	.	.	.	.	.	R	.	T	Q	.	A	A	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
AY362724.1 X (vv+) USA	.	K	D	.	.	.	.	.	.	R	.	T	Q	.	A	A	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
AY362714.1 549 (vv) USA	.	K	D	.	.	.	.	.	.	R	.	T	Q	.	A	A	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
AF493556.1 G2 (vv) China	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.
AY362719.1 New (vv+) USA	.	K	D	.	.	.	.	.	.	R	.	T	Q	.	A	.	.	.	.	A	.	.	.	T	.	.	.	V	.	.	T	.
HM749325.1 tn-n2 (vv) India	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	L	.	P	.	.	.	.	T	G	.	.	.	.	.	.	.	.
HQ638155.1 XJ03 (vv) China	R	.	.	.	.	.	.	.	A	.	.	.	.	.	R	.	.	.	.	A	.	S	.	T	.	.	.	.	.	.	.	.	.
AY362711.1 573 (vt) USA	.	.	.	.	.	.	.	.	A	.	.	T	.	.	H	.	.	.	.	.	.	.	.	T	.	.	V	.	.	.	.	.	.
HM749324.1 tn-n1 (vv) India	.	K	D	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	P	.	.	.	.	T	.	.	.	.	L	.	W	.	T
AY362710.1 571 (v) USA	.	.	.	.	.	.	.	.	A	.	.	T	.	.	H	.	.	.	.	.	.	.	.	T	.	.	V	.	.	.	.	.	.
AY571784.1 ATE (vv) Hungary	.	.	.	T	R	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
MN045207.1 GaHV-2-04-TR-2018	-	.	.	.	.	L	.	S	.	.	.	T	.	.	.	.	.	.	.	.	S	.	.	T	.	.	.	.	.	.	-	-	-
MN045204-MN045212 GaHV-2 other Turkish MDVs	-	.	.	.	.	.	.	S	.	.	.	T	.	.	.	.	.	.	.	.	S	.	.	T	.	.	.	.	.	.	-	-	-

According to the phylogenetic tree based on the complete genome nucleotide sequence of the CIAV strains/isolates (Figure 2a), two phylogroups (genogroups I and II) occurred. Turkish CIAV strains/isolates were included in the same genogroup (genogroup I) with some Chinese (SD1518, HLJ15108), German (Cuxhaven), American (98D02152, 26p4), Egyptian (CIAV-EG-2, CIAV-EG-7, CIAV-EG-13, CIAV-EG-26) and Taiwanese (Isolate 9). There were some differences between the amino acid sequences of the CIAV/2011/Akcdg/TUR and CIAV/2014/Elzg-66/TUR strains (R194G, R293T, D375E, A392S, L417V for VP1; V109A for VP2; S74P for VP3). The phylogenetic analysis results of the VP1 gene of CIAVs (Figure 2b) were similar to those of the complete genome.

In sequence analysis of PCR products amplified with the H2-H5 primers, three of the 11 samples (ALV/2014/Dyrbkr-66/TUR, ALV/2015/Elzg-918/TUR, ALV/2013/Dyrbkr-65/TUR) showed high homology (98-97-99.67%) to the ALV-E subgroup, while one (MDV/2011/Akcdg/TUR) showed high homology (99.68-100%) to the ALV-K subgroup.

Accession numbers

MW219788-MWMW219798, for MDV (MEQ gene); MW219801-MW219804, for ALV (p27 gene); MW233705-MW233708, for ALV (*env* gene); MW219788-MW219798, for CIAV (complete genome).

Sequence Similarity and Identity Analysis

According to the SIAS analysis, the MEQ gene similarity rate of all MDV strains used in this study was 93.41-99.9 %. The similarity rate for MEQ of 11 Turkish MDV strains obtained from this study was

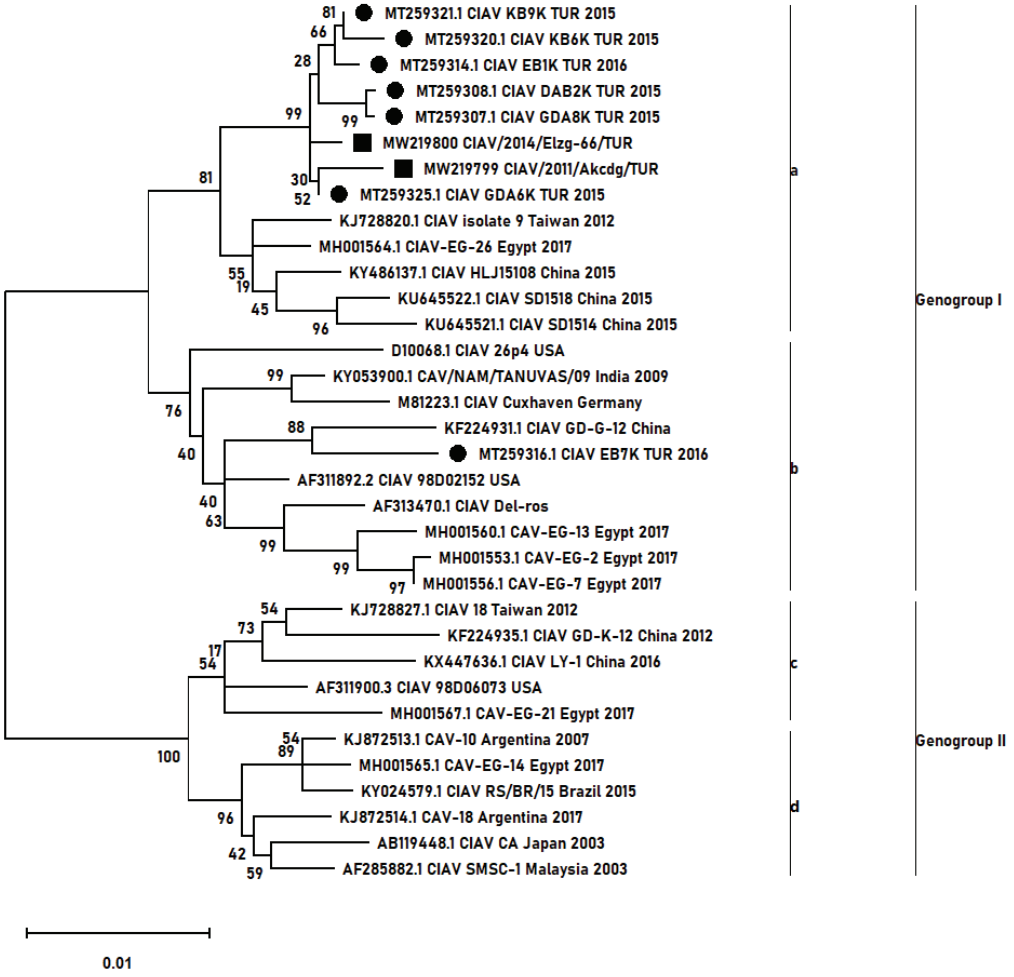


Figure 2a Phylogenetic analysis of complete genomes of CIAVs.. Phylogenetic trees were created using the Maksimun Likelihood method (1000 replicates) and Tamura Nei model by the Mega X. Filled boxes represent the Turkish CIAV strains obtained from this study, while the filled circles represent other Turkish CIAV strains

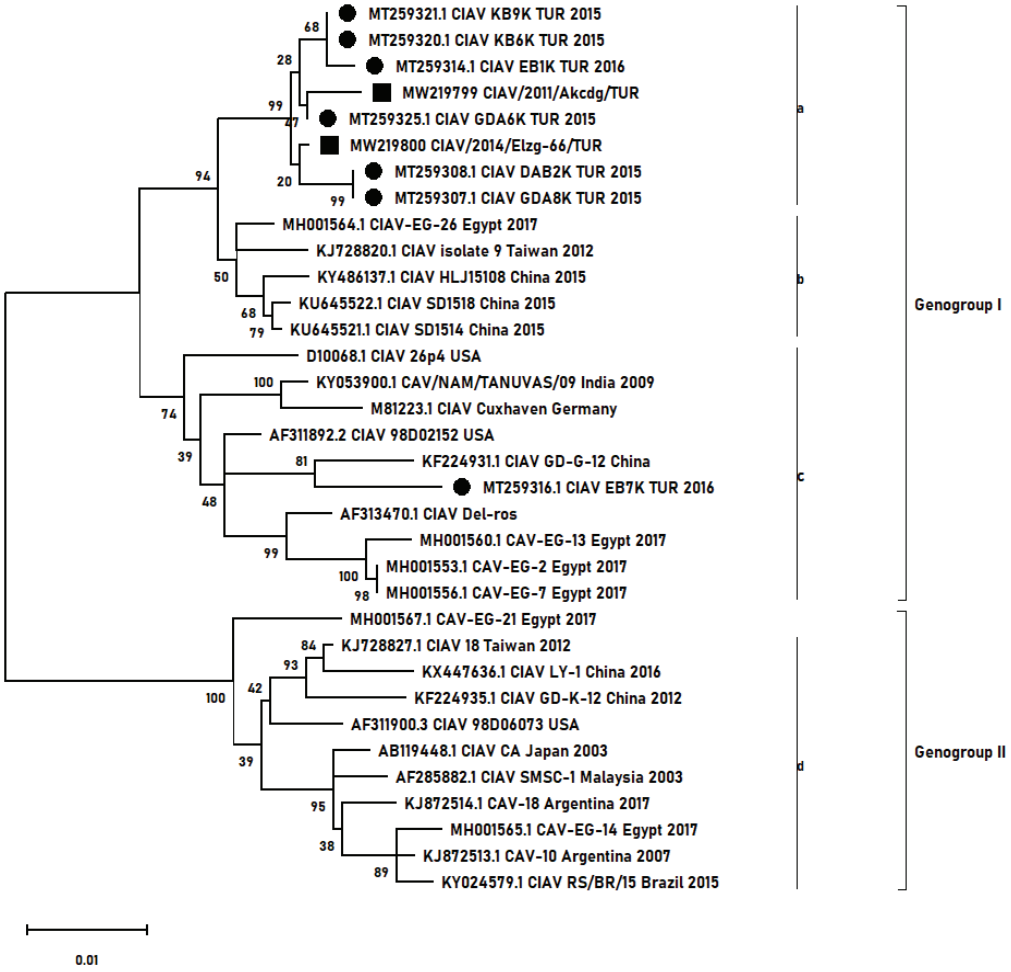


Figure 2b Phylogenetic analysis of the VP1 gene of CIAVs. Phylogenetic trees were created using the Maksimun Likelihood method (1000 replicates) and Tamura Nei model by the Mega X. Filled boxes represent the Turkish CIAV strains obtained from this study, while the filled circles represent other Turkish CIAV strains

98.68-99.9%. The similarity rate between the MEQ gene of the 11 Turkish MDV strains obtained from this study and that of the other Turkish MDV strains was 98.37-99.63%, while this ratio with vaccines was 93.41-95.57%.

According to the SIAS analysis, the similarity ratio for the complete genome nucleotide sequence of CIAV strains in this study was 93.41-99.9%. While the complete genome nucleotide sequence of the two Turkish CIAV strains obtained from this study was 99.52% similar, this ratio was 97.95-99.78% with other Turkish strains. The phylogenetic tree constructed with the variable gene region VP1 of CIAV strains was consistent with their whole genome sequence.

Recombination analysis

After recombination analysis, according to the results of four (RDP, Chimaera, Max-Chi, 3seq) of seven tests, the CIAV/2011/Akcdg/TUR strain obtained from the study was the major parent, CAV-EG-14 minor parent, and CIAV-SD1514 recombinant. According to this result, possible recombination breakpoints in the aligned sequences were between positions 503 (beginning, without gap) and 2184 (ending, without gap).

The potential recombination signal for the other CIAV strain (CIAV/2014/Elzg-66/TUR) could not be detected.

DISCUSSION

vvMDV-1 and vv+MDV-1 field strains are increasingly reported in many countries of the world, including Turkey, and cause significant yield and economic losses by affecting broilers, breeders, and com-

mercial layers (Zhuang et al., 2015; Kennedy et al., 2017; Yilmaz et al., 2020; Abayli et al., 2021, Lounas et al., 2021).

Here, we investigated oncogenic MDV-1 and ALV infections in tumor-bearing chicken flocks and immunosuppressive viral agents such as CIAV, IBDV and REV that can increase susceptibility to these infections.Virus detection by PCR and RT-PCR revealed that MDV-1 was present in all flocks examined, and no IBDV and REV were detected. In addition, co-infections of MDV-1 and ALV were detected in two of the infected flocks and MDV-1, CIAV, and ALV in the other two.

The MEQ genes of 11 MDV-1 strains contained an open reading frame of 1020 nucleotides, and no 178-bp insertions were detected, generally indicative of low virulence (Tian et al., 2011; Shamblin et al., 2005). In the phylogenetic tree of MEQ, some of the new Turkish MDV-1 strains were included in genogroup I with European, American, and Asian strains with v, vv, and vv+ pathotypes, whereas others (MDV/2019/A/Elzg/TUR, MDV/2011/Akcdg/TUR, MDV/2016/Elzg-97/TUR and MDV/2018/L/Elzg/TUR) were included in genogroup II with American strains. New Turkish MDV-1 strains had seven amino acid substitutions in the MEQ. Among these strains, those clustered in genogroup I had a 217P substitution, and those clustered in genogroup II had a 217A substitution. In addition to substitutions 217A and 217P in the C-terminal transactivation domain of MEQ, some new Turkish strains were noted to have E77 and Y80 substitutions in the bZIP domain of MEQ. There are reports that the substitutions mentioned above affect the transactivation of the MEQ (Murata et al., 2013; Murata et al., 2011).The proline-rich domain is crucial for the transactivation activity of MEQ in transformed T-cells (Liu and Kung, 2000; Qian et al., 1996).Chang et al. (2002) reported that MDV-1 strains containing a long MEQ (L-MEQ) with a proline-rich motif exhibited a higher level of transpression than MEQ variants containing the single proline-rich short meq (S-MEQ) motif. In this study, all MDV strains from different years and provinces were of average length (339 aa), and PPPP repeats in the C-terminal area of the MEQ ranged from 3 to 5. In previous studies, the virulence of MDV has been associated with the number of these PPPP repeats in the MEQ protein and reported that those with fewer (2-5) PPPP repeats have the potential to be more pathogenic (Renz et al., 2012; Shamblin et al., 2005). Similarly, some authors suggest that the most pathogenic strains have only one or two copies of 132 bp repeats, while low pathogenic strains have six or seven copies (Bradley et al., 1989; Silva et al., 2004). In this study, we found a single copy of the 132-bp repeated in all MDV strains. Interestingly, a rare substitution (P to Q at position 153) was found in the MEQ of MDV/2011/Akcdg/TUR strain obtained from MD cases in Malatya in 2011. This substitution was frequently encountered in the American reference MDV-1 strains with the pathotype vv or vv+. Considering the substitution (P to A) at position 217 of this strain, it was seen that MDV/2011/Akcdg/TUR strain had three PPPP motifs. In this context, some Turkish MDV-1 strains identified from MD cases in the past have the potential to be highly pathogenic.

Of the 11 chicken flocks in this study, two were vaccinated. Increasing MD cases in Turkey show us that especially unvaccinated chicken flocks are at high risk for MD. MDV-1's have evolved to have increased virulence over the last 40 years, acquired the ability to overcome the immune responses induced by the currently available MDV vaccines (Witter, 1997; Wozniakowski and Samorek-Salamonowicz, 2014). Here, MD cases in two HVT-vaccinated flocks prove that the vaccine's protection against existing strains is no longer sufficient. Vaccination procedures against MDV-1 in Turkey should be strictly followed and provided with bivalent HVT/SB-1 or CVI988 vaccines that provide better protection against these highly virulent field strains.

In the present study, two of the 11 MDV-1 infected flocks were also infected with CIAV and ALV agents (subgroups K or E), while the other two were ALV-E. Although CIAV is generally subclinical in older chickens (Fehler and Winter, 2001), it can affect the severity of the disease caused by immune system damage and other pathogens that co-infect birds (Davidson, 2008; Haridy et al., 2009).

The VP1 protein of CIAV has been associated with viral pathogenicity and replication. Substitutions of highly pathogenic strains (L125, E141, E144, and 394Q) were found in the VP1 sequence of new Turkish CIAV strains. Furthermore, both strains displayed the genetic character (139Q and 144Q) of VP1 of highly transmissible CIAVs (Yamaguchi et al., 2001; Natesan et al., 2006; Ducatez et al., 2006; Todd et al., 2002).In addition, this study investigated the recombination status among Turkish CIAV strains and between Turkish CIAV strains and others. Although only the CIAV/2011/Akcdg/TUR strain showed

strong recombination signals in some tests, this was insufficient.

Although there is not much information about the new subgroup ALV-K, it is known that ALV-E is an endogenous form that contributes little or nothing to tumor formation (Motta et al., 1975; Smith and Fadly, 1988). It may also affect the induction of neoplasia and other production or performance characteristics by interactions with exogenous ALV, or subgroup E recombinants that occur with the interaction may stimulate tumor formation (Crittenden and Hayward, 1980; Mays et al., 2019). It is possible that the viral co-infections detected in this study exacerbated the severity of MD. REV and IBDV infections showing similar effects were not encountered in this study.

CONCLUSIONS

Recently, MD cases have been increasing in vaccinated and unvaccinated chicken flocks in Turkey. Vaccination and strict biosecurity measures (good farm cleaning and disinfection, proper reception practices, all-in/all-out policy, accurate vaccination programs adapted to the type of bird and field situation, etc.) can significantly reduce the incidence of MD, and thereby prevent the economic loss due to the disease. The inclusion of other immunosuppressive viral agents in the fight against MD may lead to more successful results.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

PayneLN, Venugopal K(2000)Neoplastic diseases: Marek's disease, avian leukosis and reticuloendotheliosis. RevSciTech19: 544-564.

GimenoIM, Schat KA(2018) Virus-Induced Immunosuppression in Chickens. Avian Dis 62: 272-285.

OsterriederN, Kamil JP, Schumacher D,Tischer BK, Trapp S (2006) Marek's disease virus: from miasma to model. NatRevMicrobiol 4: 283–294.

FosterJE(2018)Viruses as Pathogens: Animal Viruses Affecting Wild and Domesticated Species. In: Viruses, 1st ed, Academic Press, Massachusetts: pp. 189-216.

ZhangF, Liu CJ, Zhang YP, Li ZJ, Liu AL, Yan FH, Cong F, Cheng Y (2012)Comparative full-length sequence analysis of Marek's disease virus vaccine strain 814. Arch Virol 157:177-183.

Faiz NM, Cortes AL, Guy JS, Fletcher OJ, Cimino TI, Gimeno M(2017) Evaluation of factors that affect the development of late-Marek's disease virusinduced immunosuppression: virus pathotype and host sex. Avian Pathol 6: 376-385.

QianZ, Brunovskis P, Rauscher F 3rd, Lee L, Kung HJ (1995) Transactivation activity of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected transformed T cells. J Virol 69:4037–4044.

Kung, HJ, Xıa L, Brunovskıs P, Li D, Liu JL, Lee LF (2001)Meq: An MDV-Specific bZIP Transactivator with Transforming Properties. In: Marek's Disease. Current Topics in Microbiology and Immunology, vol 255. Springer, Berlin, Heidelberg.

Chang, KS, Ohashi K, Onuma M (2002) Suppression of transcription activity of the MEQ protein of oncogenic Marek's disease virus serotype 1 (MDV1) by L-MEQ of non-oncogenic MDV1. JVetMed Sci 64:1091–1095.

ShaoH, Wang L, Sang J, Li T, Liu Y, Wan Z,Qian K,Qin A, Ye J (2017) Novel avian leukosis viruses from domestic chicken breeds in mainland China. ArchVirol162: 2073–2076.

FadlyAM, Venugopal K (2008) Leukosis/Sarcoma Group. In: Diseases of Poultry. 12th ed, Iowa State Press, Ames: pp 514–568.

VenugopalK,Howes K, Flannery DMJ, Payne LN(2000) Isolation of acutely transforming subgroup J avian leukosis viruses that induce erythroblastosis and myelocytomatosis. Avian Pathol 29:327–332.

Niewiadomska, AM, Gifford RJ(2013) The extraordinary evolutionary history of the reticuloendotheliosisviruses. PLoS Biol 11: e1001642.

WalkerMH, Rup BJ, Rubin AS, Bose HR (1983)Specificity in the immunosuppression induced by avian reticuloendotheliosis virus. Infect Immun 40: 225–235.

Adams MJ,Lefkowitz EJ, King AM, Harrach B,Harrison RL,Knowles NJ, Kropinski AM, Krupovic M, Kuhn JH,Mushegian AR, Nibert M, Sabanadzovic S, Sanfaçon H, Siddell SG, Simmonds P, Varsani A, Zerbini FM, Gorbalenya AE, Davison AJ (2016)Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses Arch Virol 161: 2921-2949.

BalamuruganV, Kataria JM (2006) Economically Important Non-oncogenic Immunosuppressive Viral Diseases of Chicken—Current Status. Vet Res Commun 30: 541–566.

SamyA, Courtillon C, Briand FX, Khalifa M, Selim A, Arafa AES, Hegazy A, Eterradossi N, Soubies SM (2020) Continuous circulation of an antigenically modified very virulent infectious bursal disease virus for fifteen years in Egypt. Infect Genet Evol 78: 104099.

Fadly AM, Witter RL, Smith EJ, Silva RF, Reed WM, Hoerr FJ, Putnam MR (1996)An outbreak of lymphomas in commercial broiler breeder chickens vaccinated with a fowlpox vaccine contaminated with reticuloendotheliosis virus. Avian Pathol 25:35–47.

KoyamaH, Suzuki Y, Ohwada Y, Saito Y(1976) Reticuloendotheliosis group virus pathogenic to chicken isolated from material infected with turkey herpesvirus (HVT). Avian Dis 20:429–434.

OngorH, Bulut H (2011)PCR based evidence of reticuloendotheliosis virus infection in chickens from Turkey. Pak Vet J 31:360-362.

YunB, Li D, Zhu H, Liu W, Qin L, Lıu Z, Wu Z, Wang Y, Qi X, Gao H, Wang X, Gao Y (2013) Development of an antigen-capture ELISA for the detection of avian leukosis virus p27 antigen. J Virol Methods 187: 278-283.

MurataS, Hashiguchi T, Hayashi Y, Yamamoto A, Matsuyama-Kato, Takasaki S, Isezaki M, Onuma M, Konnai S, Ohashi K (2013) Characterization of Meq proteins from field isolates of Marek's disease virus in Japan. Infect GenetEvol 16:137-143.

Tian M, Zhao Y, Lin Y, Zou N, Liu C, Liu P, Cao S, Wen X, Huang Y (2011) Comparative analysis of oncogenic genes revealed unique evolutionary features of field Marek's disease virus prevalent in recent years in China. Virol J 8:121.

ZhangX, Liu Y, Wu B, Sun B, Chen F, Ji J, Ma J, Xie Q (2013) Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. Sci Rep3:3519.

Sapats SI, Ignjatovıc J (2000)Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. Arch Virol 145: 773-785.

KumarS, Stecher G,Li M, Knyaz C, Tamura K (2018)MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol

Biol Evol 35: 1547-1549.
TamuraK, Nei M(1993)Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512-526.
Martin, DP, Murrell B, Golden M, Khoosal A, Muhire B (2015)RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol 1: 003.
Zhuang X, Zou H, Shi H, Shao H, Ye J, Miao J, Wu G, Qin A (2015) Outbreak of Marek's disease in a vaccinated broiler breeding flock during its peak egg-laying period in China. BMC Vet Res 11: 157.
Kennedy DA, Cairns C, Jones MJ, Bell AS, Salathé RM, Baigent SJ, Nair VK, Dunn PA, Read AF (2017). Industry-Wide Surveillance of Marek's Disease Virus on Commercial Poultry Farms. Avian Dis 61: 153-164.
Yilmaz A, Turan N, Bayraktar E, Tali HE, Aydin O, Umar S, Cakan B, Sadeyen JR, Baigent S, Iqbal M, Nair V, Yilmaz H (2020) Molecular characterisation and phylogenetic analysis of Marek's disease virus in Turkish layer chickens. Br Poult Sci. 61: 523-530.
Abayli H, Karabulut B, Ozbek R, Ongor H (2021) Timurkaan N, Tonbak S. Detection and molecular characterization of a highly oncogenic Marek's disease virus from vaccinated hens in Turkey Acta Virol 65: 212-220.
Lounas A, Besbaci M, Akkou M, Tali O (2021) Occurrence of Marek's disease in vaccinated Algerian broiler breeder flocks: A histopathological survey. Vet World 14: 3021-3027.
Shamblin CE, Greene N, Arumugaswamı V, Dıenglewıcz RL, Parcells MS (2005) Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38- and transformation antigen Meq-encoding genes: association of meq mutations with MDVs of high virulence. Vet Microbiol 102: 147-167.
Murata S, Okada T, Kano R, Hayashi R, Hashiguchi T, Onuma M, Konnai S, Ohashi K (2011) Analysis of transcriptional activities of the Meq proteins present in highly virulent Marek's disease virus strains, RB1B and Md5. Virus Genes 43: 66-71.
Liu JL, Kung HJ (2000): Marek's Disease Herpesvirus Transforming Protein MEQ: a c-Jun Analogue with an Alternative Life Style. Virus Genes 21: 51–64.
Qian Z, Brunovskis P Lee L, Vogt PK, Kung HJ (1996) Novel DNA binding specificities of a putative herpesvirus bZIP oncoprotein. J Virol 70: 7161-7170.
Renz KG, Cooke J, Clarke N, Cheetham BF, Hussaın Z, Fakhrul Islam AF, Tannock GA, Walkden-Brown SW (2012) Pathotyping of Australian isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. Avian Pathol 41: 161-176.
Bradley G, Hayashı M, Lancz G, Tanaka M, Nonoyama M (1989) Structure of the *Bam*HI-H gene family: genes of putative importance in tumor induction. J Virol 63: 2534–2542
Silva RF, Reddy SM, Lupiani B (2004) Expansion of a unique region in the Marek's disease virus genome occurs concomitantly with attenuation but is not sufficient to cause attenuation. J Virol 78: 733–740.
Witter RL (1997) Increased virulence of Marek's disease virus field isolates. Avian Dis 41: 149–163.
Wozniakowski G, Samorek-Salamonowicz AE (2014) Molecular evolution of Marek's disease virus (MDV) field strains in a 40-year time period. Avian diseases. 58: 550–557.
Fehler F, Winter C (2001) CAV infection in older chickens: an apathogenic infection? In: International Symposium on Infectious Bursal Disease and Chicken Infectious Anemia, Rauischolzhausen, Germany, pp. 391–394.
Davidson I (2008) The synergistic disease severity and virus shedding in MDV and CAV infected chickens depends on the relative time of coinfection. In: Proc. 15th World Veterinary Poultry Congress, the AsianPacific Poultry Health Congress (AP6), Brisbane, Australia.
Haridy M, Goryo M, Sasaki J, Okada K (2009) Pathological and immunohistochemical study of chickens with co-infection of Marek's disease virus and chicken anaemia virus. Avian Pathol 38: 469-483.
Yamaguchi S, Imada T, Kaji N, Mase M, Tsukamoto K, Tanimura N, Yuasa N (2001) Identification of a genetic determinant of pathogenicity in chicken anaemia virus. J Gen Virol 82:1233–1238.
Natesan S, Kataria JM, Dhama K, Rahul S, Bhardwaj N (2006) Biological and molecular characterization of chicken anaemia virus isolates of Indian origin. Virus Res 118:78–86.
Ducatez MF, Owoade AA, Abiola JO, Muller CP (2006) Molecular epidemiology of chicken anemia virus in Nigeria. Arch Virol 151:97–111.
Todd D, Scott AN, Ball NW, Borghmans BJ, Adair BM (2002) Molecular basis of the attenuation exhibited by molecularly cloned highly passaged chicken anemia virus isolates. J Virol 76:8472–8474.
Motta JV, Crittenden LB, Purchase HG, Stone HA, RL (1975) Low oncogenic potential of avian endogenous RNA tumor virus infection or expression. JNCI: Journal of the National Cancer Institute, Volume 55, Issue 3, pp 685–689.
Smith EJ, Fadly AM (1988) Influence of congenital transmission of endogenous virus-21 on the immune response to avian leukosis virus infection and the incidence of tumors in chickens. Poult Sci 67: 1674-1679.
Crittenden LB, Hayward WS, Hanafusa H, Fadly AM (1980) Induction of neoplasms by subgroup E recombinants of exogenous and endogenous avian retroviruses (Rous-associated virus type 60). J Virol 33: 915–919.
Mays JK, Black-Pyrkosz A, Mansour T, Schutte BC , Chang S, Dong K, Hunt HD, Fadly AM, Zhang L, Zhang H (2019) Endogenous Avian Leukosis Virus in Combination with Serotype 2 Marek's Disease Virus Significantly Boosted the Incidence of Lymphoid Leukosis-Like Bursal Lymphomas in Susceptible Chickens. J Virol 93: e00861-19.