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Detection and molecular characterization of a highly oncogenic Marek's disease virus from vaccinated hens in Turkey

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Summary. – Marek's disease (MD) is a highly contagious neoplastic disease of chickens associated with economic losses, often due to visceral lymphomas. The etiological agent is MD virus serotype 1 (MDV-1), also called Gallid alphaherpesvirus 2 (GaHV-2). Despite intensive vaccination, MDV is constantly evolving and maintaining its presence in the world. The aim of this study was to genetically analyze a highly oncogenic MDV/Tur/2019 strain obtained from a poultry farm in Turkey's Elazig province in 2019. Genes associated with viral pathogenicity and oncogenicity Marek's EcoRI-Q-encoded protein (MEQ), phosphoprotein-38 (pp38), and viral interleukin 8 (vIL-8) were selected for this purpose. The vIL-8 nucleotide sequence showed high similarity (100% identity) to some European (EU-1, Polen 5) and Asian (03 India, GADVASU-M2) MDV strains. The pp38 nucleotide sequence showed high similarity (100% identity) to some American (CU-2, JM/102W, RB1B) and European (MD70/13, ATE2539) MDV strains. There were no disrupted four-proline molecules (PPPP) within the transactivation domain of the MEQ. However, according to phylogenetic results, the MDV/Tur/2019 strain was included in cluster 2a alongside European MDV strains (Polish, Hungarian, Italian) with very virulent and very virulent plus pathotypes. In conclusion, we believe that the MDV/Tur/2019 strain obtained from turkey herpesvirus (HVT)-vaccinated chickens has a very virulent or very virulent plus pathotype. Although this result provides some clues regarding the virulence of this strain, *in vivo* studies are needed to achieve exact pathotyping. Further, combination of HVT with the CVI988 strain should be used for vaccination to provide the best protection, as highly pathogenic MDV strains can break sterile immunity against the HVT vaccine.

Keywords: GaHV-2; Marek's disease; oncogenes; Turkey

Introduction

Marek's disease (MD) causes serious economic losses in the poultry industry worldwide (Morrow and Fehler, 2004). Classification of the MD virus (MDV) is based on virulence factors and serotypes. There are three serotypes of MDV: serotype 1 (Gallid herpesvirus 2, GaHV-2) comprises pathogenic strains and attenuated variants; serotype 2 (Gallid herpesvirus 3, GaHV-3) is a group of very low pathogenicity; and serotype 3 (Meleagrid herpesvirus 1, MeHV-1) is the turkey herpesvirus (HVT), which is preferred as a vaccine against MD because it is non-pathogenic in chickens. There is also an additional classification for serotype 1 that divides the viruses into four pathotypes (mild, virulent, very virulent, and very virulent plus) based on their virulence (Lopez-Osorio *et al*., 2017). The etiological agent of MD virus serotype 1 (MDV-1) or Gallid alphaherpesvirus 2 (GaHV-2) belongs to the genus *Mardivirus* in the subfamily *Alphaherpesvirinae* (International Committee on Taxonomy of Viruses, 2005).

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Abbreviations: ALV = avian leucosis virus; CAV = chicken anemia virus; HVT = turkey herpesvirus; IBDV = infectious bursal diseases virus; IL = interleukin; MD = Marek's disease; MDV = Marek's disease virus; MDV-1, -2, 3 = Marek's disease virus serotype 1, 2, 3; MEQ = Marek's EcoRI-Q-encoded protein; PPPP = four proline molecules; REV = reticuloendotheliosis virus

This virus causes malignant lymphomas in the natural host, chickens, within a few weeks of infection (Lee *et al*., 2000; Calnek, 2001). The early pathogenesis of MD involves an initial lytic infection of the B cells followed by a latent infection of the activated CD4 T cells, leading to the destruction of the B and T cells, bursa fabricius and thymus atrophy, and transient immunosuppression (Calnek, 2001). The last period of MDV infection is characterized by reactivation of the virus with a second cycle of lytic infection, induction of T-cell lymphomas, and permanent immunosuppression (Schat and Nair, 2008). T-cell lymphomas are mostly localized in the visceral organs (the kidneys, spleen, liver, gonads, and proventriculus), peripheral nerves, skin, and muscles (Couteaudier and Denesvre, 2014). The MDV contains more than 200 genes as well as major latency/transformation Marek's EcoRI-Q-encoded protein (MEQ), major lytic antigen phosphoprotein-38 (pp38), and viral interleukin 8 (vIL-8); these genes are frequently associated with oncogenicity and pathogenicity (Lupiani *et al*., 2004; Cui *et al*., 2005; Gimeno *et al*., 2005). Major lytic antigen pp38 maintains the transformation of T lymphocytes by preventing their apoptosis (Gimeno *et al*., 2005). The vIL-8 protein plays an important role in early cytolytic infection and in the switch of the infection from B to T lymphocytes (Abdul-Careem *et al*., 2008). The MEQ gene encodes an oncoprotein (339 amino acids) with the N-terminal basic leucine zipper (bZIP) domain and the C-terminal proline-rich transactivation domain (Jones *et al*., 1992; Qian *et al*., 1995; Jarosinski *et al*., 2006).

In previous studies, distinct polymorphisms and point mutations in the MEQ gene and decreases in the number of four-proline molecules (PPPP) in proline-rich repeats of the MEQ transactivation domain have been found to be strong indicators of virulence (Shamblin *et al*., 2004; Renz *et al*., 2012). The MEQ gene in attenuated and mild virulent MDV strains is 59 amino acids (177 nucleotide sequences) longer than the MEQ gene in oncogenic strains; this difference is probably responsible for the non-oncogenicity of the former strains. Consequently, deletions of pp38, vIL-8, and MEQ genes result in significant reduction in tumor formation and pathogenicity (Cui *et al*., 2004; Jarosinski *et al*., 2006; Engel *et al*., 2012).

Herein, we aimed to provide a genetic analysis of genes associated with the pathogenicity of a highly oncogenic MDV/Tur/2019 strain obtained from a poultry farm in Turkey's Elazig province in 2019.

Materials and Methods

Animal information. The disease under study (MD) broke out in a poultry farm in Turkey's Elazig province in 2019. This poultry farm had 318 Blue Cochin chickens of different ages. Within two weeks, 38 of the 79 affected chickens died; others were severely affected by the disease, exhibiting non-specific clinical symptoms such as anorexia, weight loss, and lethargy. Most of the affected chickens were three to four months old. We learned from the farmer that all the chickens were vaccinated with the HVT/vector laryngotracheitis (HVT-LT) commercial vaccine at the age of one day.

Tissue preparation. Tissue specimens were collected, fixed in a 10% neutral buffered formalin solution, and then placed in standard tissue processing cassettes (Isolab, Germany). After the specimens were washed under running tap water for approximately two hours, they were passed through an alcohol, xylol, and paraffin series in an automatic tissue processing device (Leica, Germany) and then paraffin-embedded on a tissue embedding device (Leica, Germany). Paraffin blocks were taken into a rotary microtome (Leica, Germany), and three-micron-thick sections were taken to charged (+) slides (Thermo Fischer Scientific, USA). The sections were then stained with hematoxylin-eosin in an automatic tissue staining machine (Leica, Germany) and examined under light microscope (Olympus, Japan).

DNA extraction and polymerase chain reaction. Tumoral tissue sections were homogenized in PBS. The resulting homogenate was stored at -80°C, freeze-thawed several times, and then centrifuged at 1,500 x*g* for 15 min. Viral nucleic acid was obtained from the supernatant using a QIAamp MinElute Virus Spin Kit (Qiagen, Germany) according to manufacturer's instructions. For the detection of avian leukosis and reticuloendotheliosis caused by oncogenic viruses, we followed the polymerase chain reaction (PCR) procedure used by Ongor and Bulut (2011). Reference primers and PCR procedures were used to detect immunosuppressive agents, such as infectious chicken anemia (Zhang *et al*., 2013) and infectious bursal disease virus (IBDV; Sapats *et al*., 2000). For the detection of MD, MEQ-specific primers were preferred. The MDV DNA was then used for the amplification of the 132 repeat, pp38, and vIL-8 regions (Tian *et al*., 2011). The obtained amplicons were analyzed by electrophoresis (110 V/40 min) in 1.5% (w/v) agarose gel stained with ethidium bromide (EtBr).

Sequencing and phylogenetic analysis. The expected amplicons for the MEQ, pp38, and vIL-8 genes were sequenced in the Macrogen laboratory (Macrogen Corporation, Netherlands). An ABI 3730XL Sanger sequencing device (Applied Biosystems, USA) and an ABI BigDye Terminator v3.1 Cycle Sequencing Kit were used at this stage. The bidirectional sequence data were aligned with the ClustalW program and verified with BLAST software. The sequences obtained in this study were submitted to GenBank. Multiple nucleotide sequence alignments were performed using Mega X software for comparison of the deduced pp38, vIL-8, and MEQ amino acid sequences with the vaccine strains and wild MDV strains deposited in the GenBank database. Then, phylogenetic trees were constructed for pp38 and vIL-8 using the maximum likelihood method algorithm (bootstrap, 1,000 replicates) with the Tamura Nei model. A third phylogenetic tree was generated for MEQ using the maximum

Fig. 1

Gross pathological findings in the abdominal organs of affected chickens in postmortem examination
Liver and spleen, are enlarged filled the entire abdomen and contain multifocal nodules of varying size **(a, b, c,)**. Multifocal nodules (arrow) over the epicardial surface of the heart **(d).**

likelihood algorithm (bootstrap, 1,000 replicates) with the JTT matrix-based model.

Results

Gross pathological findings

In the postmortem examination of the affected chickens, liver lesions were observed in all ten chickens; in addition, spleen lesions were observed in six chickens. In some cases, these were accompanied by lesions of the lung, proventricle, heart, and less often the intestine.

The liver and spleen lesions were usually numerous nodular structures of varying sizes (0.3 to 1.5 cm) and white in color. The nodular structures in other organs were found to be more miliary. Some livers and spleens showed only diffuse enlargement without any apparent nodular focal lesions. There were no macroscopic lesions in the peripheral nerves.

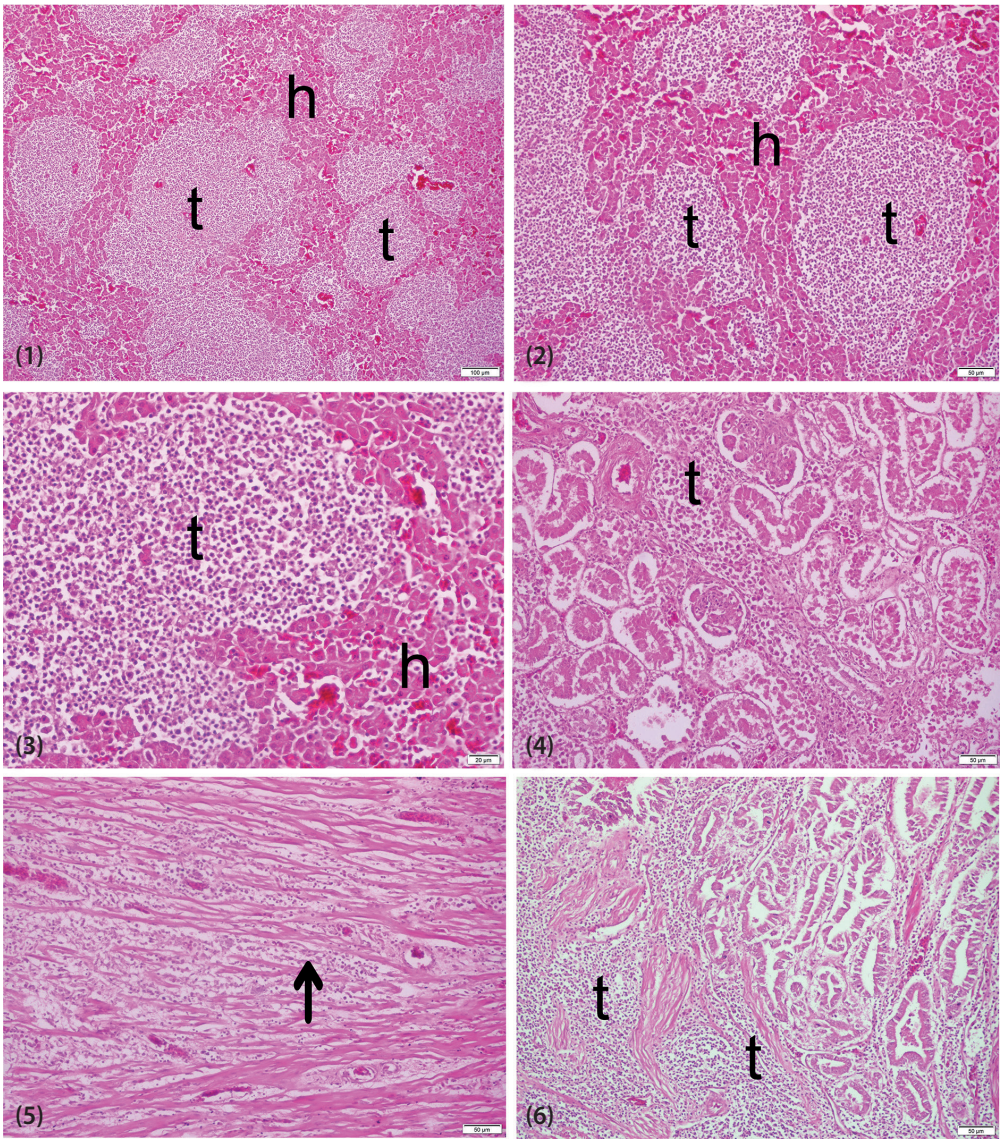

Fig. 2

Histopathological examination of various organs of affected chickens

Liver sections (**1**, **2** and **3**) showing tumoral foci consisting of atypical lymphocytes in hepatic perenchyma (**t**) and decreased number of hepatocytes (**h**). Kidney section (**4**), tumor cells (**t**) between the renal tubules and glomeruli. Heart section (**5**), tumor cells between the muscle fibers (arrow). Proventriculus (**6**), tumor cells (**t**) between proventricular glands. (Magnifications: **1**, **4**, **6**: 100x; **2**, **5**: 200x; **3**: 400x).

Representative gross pathological findings in the abdominal organs of affected chickens in postmortem examination are shown in Fig. 1.

Microscopic findings

The histopathological examination revealed lymphoproliferative foci of various sizes in the liver, spleen, lungs, heart, kidneys, proventriculus, and intestines. These cells included lymphoblasts and lymphocytes together with plasma cells and macrophages. Atypical and pleomorphic tumor cells were located multifocally in some areas, ranging from small foci to nodular structures in the liver, lungs, and spleen. Tumor cells were found between the muscle fibers in the heart, between the renal tubules and the glomeruli in the kidneys, and in the mucosa and submucosa of the proventriculus and intestines. Histopathological changes were not observed in the central and peripheral nervous systems.

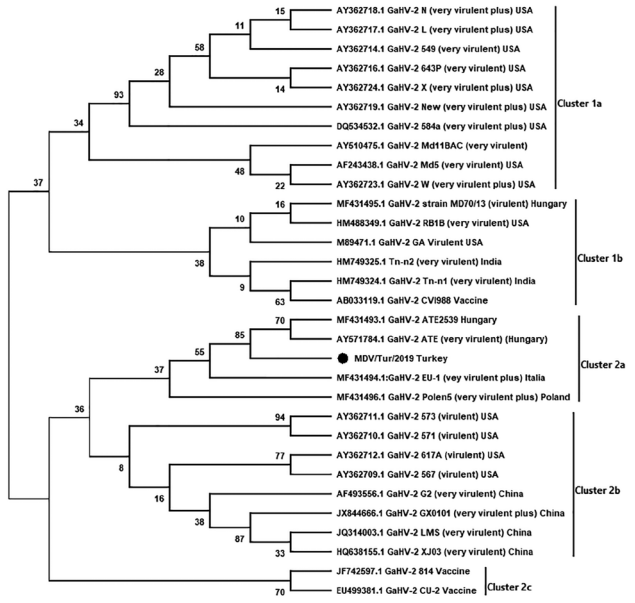

Fig. 3

Phylogenetic relationship between MDV strains with different pathotypes from different countries (GenBank) based on MEQ amino acid sequence

The phylogenetic tree was constructed using MEGA version X by maximum likelihood method (bootstrap, 1000 replicates) and JTT matrix-based model. The black-filled circle represents the Marek's disease virus strain obtained in this study.

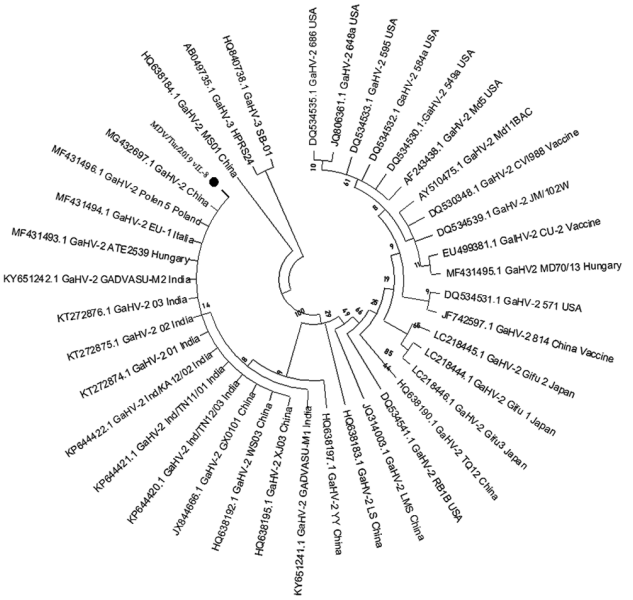

Fig. 4

Phylogenetic analysis of the vIL-8 gene of MDV/Tur/2019 strain and other reference MDVs based on nucleotide sequences

The phylogenetic tree was constructed using MEGA version X by using the Maximum-likelihood method algorithm (bootstrap, 1000 replicates) with Tamura Nei model. The black-filled circle represents the Marek's disease virus strain obtained in this study

Lymphoproliferative foci in histopathological examination of various organs are shown in the Fig. 2.

Sequencing and phylogenetic analysis

Sequencing data for the MEQ, vIL-8, and pp8 genes of the MDV/Tur/2019 strain were submitted to GenBank with accession numbers MN956505, MN956506, and MN956507, respectively.

PCR targeting 132-bp repeat regions

We found that the MDV/Tur/2019 strain had only one copy of the 132 base tandem repeats; thus, the size of the amplicon obtained by PCR with 132 repeat primers was 314 bp.

Amino acid sequence analysis of the MEQ gene

The PCR product of a size expected for the MEQ gene (1,062 bp) was obtained for all clinical samples tested, but no *long-MEQ* gene (L-MEQ) was detected. The amino acid sequence for the MEQ of the MDV/Tur/2019 strain was compared with those of MDV strains from different geographic locations and different pathotypes in the GenBank database, and a phylogenetic tree was created (Fig. 3). Based on the results of this comparison, the MDV/Tur/2019 strain was included in the cluster 2a alongside European MDV strains (Polish, Hungarian, Italian) with very virulent and very virulent plus pathotypes. Based on the same results, very virulent and very virulent plus MDV strains from the USA were included in cluster 1a, while virulent American strains and very virulent Chinese strains were included in cluster 2b. Some Hungarian, Indian, and American MDV strains were included in cluster 1b along with the CVI988 vaccine strain. Through multiple amino acid alignments, amino acid differences were found in 21 different loci between the MDV/Tur/2019 and GaHV-2 strains referenced in Table 1. Some of these were K77E, D80Y, A88T, Q93R, V(A)115L, R119C, T139A, Q153P, A(H/R)176P, A180T, and R217P.

The MEQ amino acid sequence similarity between the MDV/Tur/2019 strain and the GaHV-2 strains selected for this study were 95.7% to 99.66%.

Nucleotide sequence analysis of vIL-8 and pp38 genes

According to the PCR results, amplification products of 887 bp and 1,006 bp in length were obtained for vIL-8 and

Table 1. Multiple alignment of MEQ amino acid sequence of Turkish and reference strains of MDV

Accession numbers	Strains/isolates (pathotype)	Basic region					Leucine Zipper				Repression/transactivation domain											
							1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	3
		4	7	8	8	9	0	1	1	1	3	5	7	8	1	1	5	6	7	8	8	3
		8	7	0	8	3	1	0	5	9	9	3	6	0	7	8	4	5	7	1	7	9
MN956505	MDV/Tur/2019	F	E	Y	T	R	K	C	L	C	A	P	P	T	P	S	H	V	A	W	Y	T
M89471	GaHV-2 GA (v)	.	K	D	A	Q	.	.	V	.	T	.	.	.	.	.	N	.	.	.	.	.
MF431493	GaHV-2 ATE2539 (vv)	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.
MF431495	GaHV-2 strain MD70/13 (v)	.	K	D	A	Q	.	.	V	.	T	.	.	.	.	.	.	.	.	.	.	.
AY362712	GaHV-2 617A (v)	.	.	.	A	Q	.	.	V	R	T	.	.	.	R	.	.	.	.	.	.	.
AY362711	GaHV-2 573 (v)	.	.	.	A	Q	.	.	A	.	T	.	H	.	.	.	.	.	.	C	.	.
AY362709	GaHV-2 567 (v)	S	.	.	A	Q	.	.	V	R	T	.	.	.	R	.	.	.	.	.	.	.
AY362710	GaHV-2 571 (v)	.	.	.	A	Q	.	.	A	.	T	.	H	.	.	.	.	.	.	C	.	.
AY571784	GaHV-2 ATE (vv)	.	.	.	.	.	R	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.
AY510475	GaHV-2 Md11BAC (vv)	.	K	D	A	Q	.	.	V	.	T	.	.	.	R	.	.	.	.	.	.	.
AF493556	GaHV-2 G2 (vv)	.	.	.	A	Q	.	.	A	.	T	.	.	.	R	.	.	.	.	.	.	.
HM488349	GaHV-2 RB1B (vv)	.	K	D	A	Q	.	.	V	.	T	.	.	.	.	.	.	.	.	.	.	.
HQ638155	GaHV-2 XJ03 (vv)	.	.	.	A	Q	.	.	A	.	.	.	R	.	R	.	.	.	.	.	.	.
MF431494	GaHV-2 EU-1 (vv)	.	.	.	.	Q	.	.	V	.	T	.	.	.	.	.	.	A	.	.	.	.
JQ314003	GaHV-2 LMS (vv)	.	.	.	A	Q	.	.	A	.	.	.	R	.	R	.	.	.	.	.	.	.
AF243438	GaHV-2 Md5 (vv)	.	K	D	A	Q	.	.	V	.	T	.	.	.	R	.	.	.	.	.	.	.
AY362714	GaHV-2 549 (vv)	.	K	D	A	Q	.	.	V	R	T	Q	A	A	R	.	.	.	.	.	.	.
HM749324	GaHV-2 Tn-n1 (vv)	.	K	D	A	Q	.	.	V	.	T	.	.	.	.	.	.	.	.	.	N	N
AY362716	GaHV-2 643P (vv)	.	K	D	A	Q	.	.	V	R	T	Q	A	A	R	.	.	.	V	.	.	.
HM749325	GaHV-2 Tn-n2 (vv)	.	K	D	A	Q	.	.	V	.	T	.	.	.	.	.	.	.	.	.	.	.
DQ534532	GaHV-2 584a (vv+)	.	K	D	A	Q	.	.	V	R	T	Q	A	.	R	.	.	.	.	.	.	.
MF431496	GaHV-2 Polen5 (vv+)	.	.	.	A	Q	.	S	V	.	T	.	.	.	.	F	.	.	.	.	.	.
JX844666	GaHV-2 GX0101 (vv+)	.	.	.	A	Q	.	.	A	.	.	.	R	.	R	.	.	.	.	.	.	.
AY362723	GaHV-2 W (vv+)	.	K	D	A	Q	.	.	V	.	T	.	.	.	R	.	.	.	.	.	.	.
AY362719	GaHV-2 New (vv+)	.	K	D	A	Q	.	.	V	R	T	Q	A	.	R	.	.	.	.	.	.	.
AY362718	GaHV-2 N (vv+)	.	K	D	A	Q	.	.	V	R	T	Q	A	A	R	.	.	.	.	.	.	.
AY362717	GaHV-2 L (vv+)	.	K	D	A	Q	.	.	V	R	T	Q	A	A	R	.	.	.	.	.	.	.
AY362724	GaHV-2 X (vv+)	.	K	D	A	Q	.	.	V	R	T	Q	A	A	R	.	.	.	.	.	.	.

Amino acid residues identical to codes of MDV/Tur/2019 are indicated by dots (.) and others by letters. Pathotypes of vv+MDV, very virulent plus MDV; vvMDV, very virulent MDV; vMDV, virulent MDV.

pp38, respectively. The nucleotide sequence similarity between the MDV/Tur/2019 strain and the GaHV-2 strains selected for this study were 98.96% to 100% and 96.29% to 100% for vIL-8 and pp38, respectively. The phylogenetic tree constructed based on to the nucleotide sequence analysis of the vIL-8 and pp38 genes is shown in Figs 4 and 5, respectively.

Detection of other viruses

The PCR amplification results for the genes of the reticuloendotheliosis virus (REV) and avian leucosis virus (ALV; subgroups A-B-C-J) were negative for all samples, while amplification of the 310-bp product was detected in ALV subgroup E. According to the PCR results, chicken anemia virus (CAV) and IBDV nucleic acids were not detected in the clinical samples.

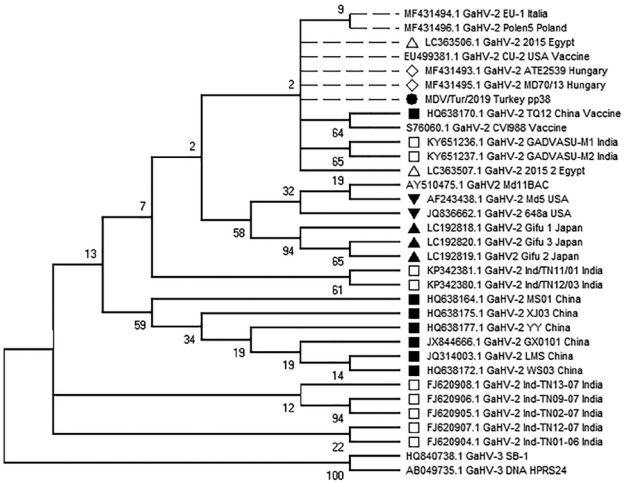

Fig. 5

Phylogenetic analysis of the pp38 gene of MDV/Tur/2019 strain and other reference MDVs based on nucleotide sequences
The phylogenetic tree was constructed using MEGA version X by using the Maximum-likelihood method algorithm (bootstrap, 1000 replicates) with Tamura Nei model. The black-filled circle represents the Marek's disease virus strain obtained in this study.

Discussion

This study examined an MD outbreak that occurred in Turkey in 2019. This disease progressed with 24.8% morbidity and 12% mortality in Cochin chickens. It was particularly noteworthy that a highly oncogenic MD outbreak occurred in chickens vaccinated with HVT. This study also involved the molecular characterization of genes related to the oncogenicity and pathogenicity of the MDV strain obtained from Cochin chickens.

According to the results of the similarity analysis, the vIL-8 nucleotide sequence of the MDV/Tur/2019 strain was 100% similar to several Chinese (GaHV-2 China), Polish (Polen 5), Italian (EU-1), Hungarian (ATE2539), and Indian (03 India, GADVASU-M2) strains. The pp38 nucleotide sequence of the MDV/Tur/2019 strain was 100% similar to several Hungarian (MD70/13, ATE2539) and American (CU-2, JM/102W, RB1B) strains. Interestingly, the MEQ amino acid sequence of the MDV/Tur /2019 strain was found to be very similar to that of the Hungarian strain. When the amino acid sequences of the MDV/Tur/2019 and Hungarian strains were examined, only Y115V changes were observed in strain ATE2539 (99.67% identity, MF431493.1). In addition, a K101R change was observed in the MEQ amino acid sequence of ATE (99.33% identity, AY571784.1), another Hungarian strain of MDV. The MEQ amino acid sequence similarity ratio was lowest between the MDV/Tur/2019 and CVI988 strains (95.7%) compared to the other MDV strains selected for this study. The MEQ amino acid sequence of the MDV/Tur/2019 strain contained five intact PPPP motifs. Previous reports have stated that changes in PPPPs in the MEQ amino acid sequence are inversely related to virulence (Shamblin *et al*., 2004; Renz *et al*., 2012) Although there were no changes in the MEQ PPPPs in the MDV/Tur/2019 strain, this strain was included in cluster 2a along with very virulent plus Italian and Polish strains as well as a very virulent Hungarian strain. Some authors have suggested that the most pathogenic strains have only one or two repeats of the 132-bp band, while mild strains have between six and seven repetitions (López-Osorio *et al*., 2017). In the present study, we found that the MDV/Tur/2019 strain had a single copy of 132 tandem repeats.

With the use of live vaccines, MD has been largely controlled and disease-related losses have been greatly reduced worldwide (Witter, 1997). However, the virulence of the MDV constantly increased toward the end of the 20th century, leaving the poultry industry desperate.

It is widely believed that live vaccines are effective in the evolution of MDV and cannot prevent infection and shedding of pathogenic MDV in chickens, thus causing prolonged infections (Witter, 2001; Davison and Nair, 2005; Wozniakowski *et al*., 2011; Hunt and Dunn, 2015; Zhang *et al*., 2015).

Vaccines based on HVT, which were generally used alone in previous years, are now used in combination with CVI988 and SB-1 vaccines (Witter and Lee, 1984; Witter, 2001). The combination of HVT and CVI988 vaccines is the most effective approach against MD (Gong *et al*., 2014), but MDVs of the very virulent plus (+) pathotype can occasionally break sterile immunity (Davison and Nair, 2005; Hunt and Dunn, 2015; Zhang *et al*., 2015). We learned that some of the Cochin chickens suffering from MD in this study were vaccinated with the HVT-LT vaccine at the age of one day. The majority of chickens affected by MD were three to four months old. The HVT-LT vaccine is used frequently in Turkey, especially for broiler chickens. The main purpose of these commercially available vaccines, which are administered subcutaneously, is to protect against infectious laryngotracheitis virus infection. Although the pathotype of the MDV/Tur/2019 strain in this study remains uncertain, an examination of the MEQ phylogenetic tree indicated that it is of a very virulent or very virulent plus pathotype. HVT vaccines are insufficient for protection against highly virulent strains of MDV. Combinations of HVT and CVI988 strains should be used to provide the most effective protection against MD.

Potential underlying causes of serious MDV infections leading to large financial losses include IBDV, chicken infectious anemia virus, adenovirus, parvoviruses (chicken parvovirus and turkey parvovirus) and retroviruses (ALV, Rous sarcoma virus, and REV). Some of these (e.g., REV)

may cause mixed infections simultaneously with pathogenic MDV strains; however, others (e.g., IBDV and CAV) may have indirect effects on MDV outbreaks by reducing the effectiveness of protective MDV vaccines (Cheng *et al*., 2011; Niewiadomska and Gifford, 2013; Tarasiuk *et al*., 2013; Zhang *et al*., 2019). In the past, MDV (Wozniakowski *et al*., 2015) and IBDV (Li *et al*., 2016) vaccines have reportedly been contaminated with REV. Furthermore, Newcastle disease virus and avian poxvirus vaccines have reportedly been contaminated with chicken infectious anemia virus (Li *et al*., 2017). However, the clinical samples of the chickens examined in the present study were negative for REV, ALV (subgroups A, B, C, D, and J), CAV, and IBDV. Although the endogenous form of ALV (subgroup E) was detected in all clinical samples, it was not always associated with the disease.

Although the results of this study provided some clues to the virulence of the MDV/Tur/2019 strain, *in vivo* studies are needed to achieve exact pathotyping of MDV. Also, the negativity of other viral infections (especially immunosuppressive viral agents) should be confirmed in future studies, as in the present study. Finally, combinations with the CVI988 strain should be used to provide the best protection, as high-pathogen MDV strains can break sterile immunity against the HVT vaccine.

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