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Marek's Disease in Broiler Farms, Iran, 2021: The Phylogenetic Study

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Abstract: Marek's disease virus (MDV) is a highly cell-associated oncogenic alphaherpesvirus that causes chicken T-cell lymphoma. MD is currently controlled by vaccination; however, MDV strains tend to develop increased virulence. Distinct diversity and point mutations are present in the *Meq* proteins, the major oncoproteins of MDV, suggesting that changes in protein function induced by amino acid substitutions might affect MDV virulence. This study sampled 30 commercial broiler flocks from different Provinces (10 spleens from each flock) at slaughter. Gallid alphaherpesvirus 2 was identified in PCR (using gb primer) of spleen samples of 11 flocks (36.67%). Two provinces, Azerbaijan Gharbi (44%) and Golestan (25%), recorded the highest and lowest infection rates. The oncogene *Meq* of some positive samples was amplified by PCR and sequenced. MDV strains detected in this study could be put in three branches, with molecular features consistent with virulent and very virulent previously identified MDV. UT-PCR9303 was located with an Iraqi isolate. UT-PCR-9231 had high homology with an Iranian MDV sequence detected from a layer farm with MD. UT-PCR-9380 was located with vv MDV from Japan and Colombia. Therefore, the relatively high rate of *Meq* in the unvaccinated broiler farms constitutes support for vaccination. These findings provide the basis for molecular surveillance and further study of MDV mutants and strategies for managing MD in Iran.

Keywords: Marek Disease, Iran, Broiler, Phylogenetic Analysis, *Meq*

1. Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by oncogenic (serotype 1) strains of Marek's disease herpesvirus (MDV). MDV (family, *Herpesviridae*; subfamily, *α-Herpesvirinae*; genus, *Mardivirus*; species, *Gallid alphaherpesvirus* 2) is the causative agent of Marek's disease (MD), which manifests as malignant lymphomas in infected chickens [1]. Attenuated MDV strains and the naturally non-oncogenic *Gallid alphaherpesvirus* 3 and *Meleagrid alphaherpesvirus* 1 (turkey herpesvirus, HVT) have been used as monovalent or multivalent vaccines. Modified live vaccines are the cornerstone of protection against MD [2]. CVI988 is considered the most protective vaccine currently available, an attenuated MDV strain, which has been introduced in many countries. However, the virulence of MDV field strains tends to increase, and the virulence of GaHV-2 isolates has shifted over the years from mild (m) to virulent (v), very virulent (vv), and very virulent + (vv+) [1]. What is known is that *Meq*, the only known oncoprotein encoded by an *alphaherpesvirus*, is necessary and plays an essential role in the transformation at the individual cell level. The *Meq* gene encodes the *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. The last 33 carboxy-terminal amino acids (aa) are essential for transcriptional transactivation, whereas the number of proline-rich repeats (PRR) in the transactivation domain seems to be related to the repression of transcription [3]. The gene *Meq* is polymorphic, with various recognized sizes: long-*meq* (L-*meq*), *meq*, short-*meq* (S-*meq*), and very short-*meq* (VS-*meq*); these encode Meq protein isoforms with 399, 339, 298, and 247 aa, respectively. These different length Meq isoforms are due to insertions or deletions in the transactivation domain, resulting in a variable number of PRR. This number and specific point mutations in the PRR appear to correlate with GaHV2 virulence [4, 5]. High genetic diversity has been reported for the *Meq* gene despite the relatively low evolutionary rates of change that commonly characterize dsDNA viruses. The data reveal that the *Meq* gene sequence evolves faster than most dsDNA viruses and is comparable to RNA viruses' evolutionary rate [6].

The current study was designed to carry out the molecular detection of MDV in broilers of Iran and phylogenetic analysis of MDV based on the *Meq* gene sequencing.

2. Material and Methods

2.1. Samples

The examined samples (spleens) were collected in 2021 from commercial broiler flocks at slaughters (42-48 day-old) in four provinces of Iran, including Azarbyjan-Gharbi, Isfahan, Gilan, and Golestan. Ten spleens per flock were the source of the analyzed samples. The samples were stored at -20°C until DNA extraction.

2.2. DNA Extraction

Total DNA was extracted using Sinaclon Extraction (Sinaclon, Iran) from 200 µL of homogenates of the spleen taken from chickens following the manufacturer's instructions and stored at -20°C until use.

2.3. PCR for Detection of MDV

PCR amplification for MDV-1 detection using gB primer (F: TCCAATACACCAACATCAC & R: CAAGGAAACATACAGGGAC) as described by Gimeno *et al*. [7].

2.4. PCR Amplification of the Meq Gene for DNA Sequence Analysis

For the complete amplification sequence of the *Meq* gene, the protocol was used as described by Wajid *et al.* [8].

2.5. Bioinformatic Analysis

The AccuPrep® PCR purification Kit (Bioneer Co., Korea) was used to purify the PCR products. Sequencing was performed with the primers (both directions) (Bioneer Co., Korea). Chromatograms were evaluated with CromasPro (CromasPro Version 1.5). After sequence editing, NCBI BLAST was done on the results and made primary identifications. In the following, a phylogenetic tree was drawn. A phylogenetic tree for the *Meq* gene was generated using the neighbor-joining method with 1000 bootstrap replicates to assign confidence levels to branches using the MEGA7 software package Kimura-2-parameter model [9]. Sequences reported in this paper were added to the GenBank database under accession numbers (MW846288, MW846289, and MW846300).

3. Results

Thirty-six percent of the studied flocks (11/30 flocks) had the virus genome traced. Among the studied provinces, Azerbaijan Gharbi (44%) and Golestan (25%) recorded the highest and lowest infection rates (Table 1).

Table 1. *The positive rate of MDV infection in Iranian commercial broiler flocks, 2021.*

No	Province	Number of Flocks	Positive	Positive (%)
1	Golestan	8	2	25.00
2	Gilan	6	2	33.33
3	Azerbaijan-Gharbi	9	4	44.44
4	Isfahan	7	3	42.86
	All	30	11	36.67

The *Meq* gene of three positive samples was completely sequenced. It has been shown that three MDVs are in at least three different groups of the phylogenetic tree (figure 1). UT-PCR9303 was located with an Iraqi isolate. UT-PCR-9231 had high homology with an Iranian MDV sequence [10] that was detected from a layer farm with MD. UT-PCR-9380 was located with vv MDV (Kgw-c2 (LC385874) & Colombia/_UDEACO-07/13 (KU058697)).

Table 2. *Sequence homology matrix for GaHV-2 isolated in this work and different GaHV-2 strains based on Amino Acid (aa) sequences of the* Meq protein.

		1	2	3	4	5	6	7	8	9	10	11
1	GaHV_Iran_UT-PCR9231_2021											
2	GaHV_Iran_UT-PCR9303_2021	99.41										
3	GaHV_Iran_UT-PCR9380_2021	99.01	99.01									
4	GaHV/Iran/Ch/H3452-1/19	99.51	98.91	98.61								
5	GaHV/Iran/Ch/H3461-2/19	100.00	99.41	99.01	99.51							
6	C12-130_vv_ (FJ436096)	99.51	99.31	99.51	99.01	99.51						
7	Iraq3A_ (KC243262)	99.33	99.89	99.21	99.21	99.33	99.21					
8	Italy_Ck_1083_18 (MK855066)	100.00	99.41	99.01	99.51	100.00	99.51	99.33				
9	686_vv+_ (AY362727)	98.91	99.11	99.11	98.51	98.91	98.81	99.33	98.91			
10	TK_vv+_ (AY362721)	99.01	99.21	99.21	98.61	99.01	98.91	99.44	99.01	99.90		
11	CVI988_ (DQ530348)	99.51	99.31	98.91	99.01	99.51	99.41	99.21	99.51	99.01	99.11	

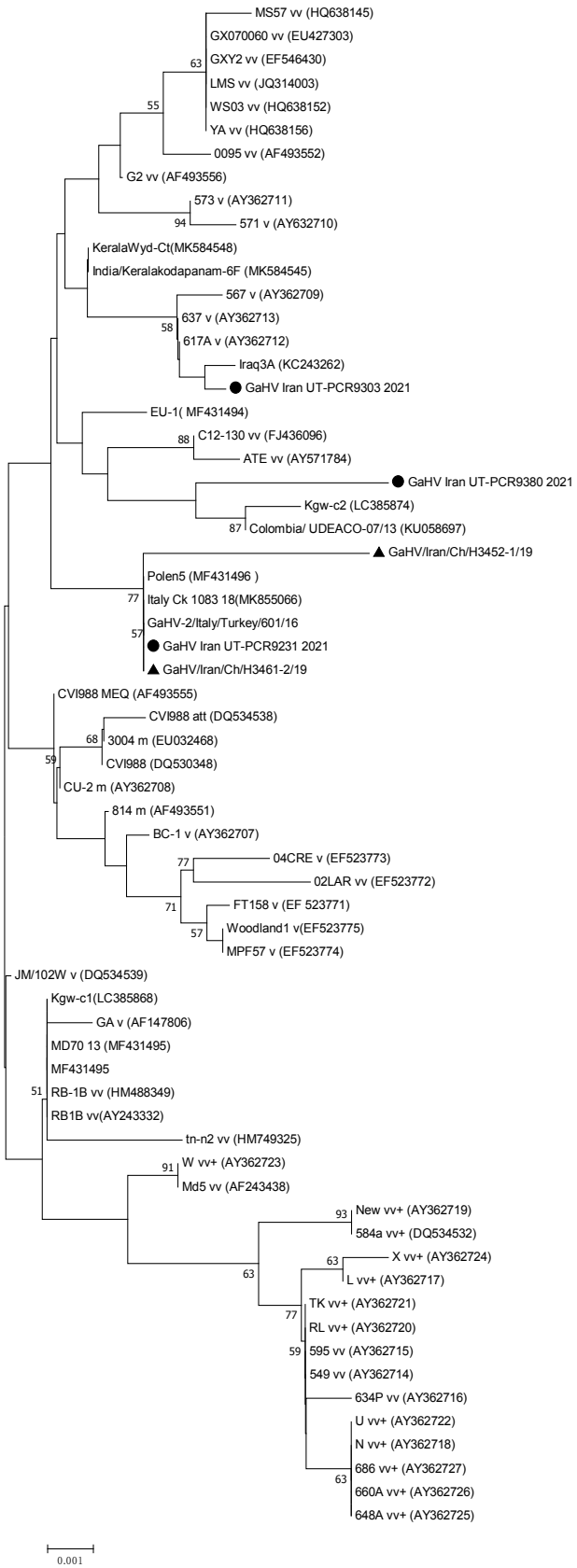

Figure 1. *The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.11984332 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 27 amino acid sequences. All ambiguous positions were removed for each sequence pair. There was a total of 399 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.*

UT-PCR-9231 had three PPPP repeats, while the others had two PPPP repeats because of the interruption within the repeats of prolines at the second positions (Figure 2).

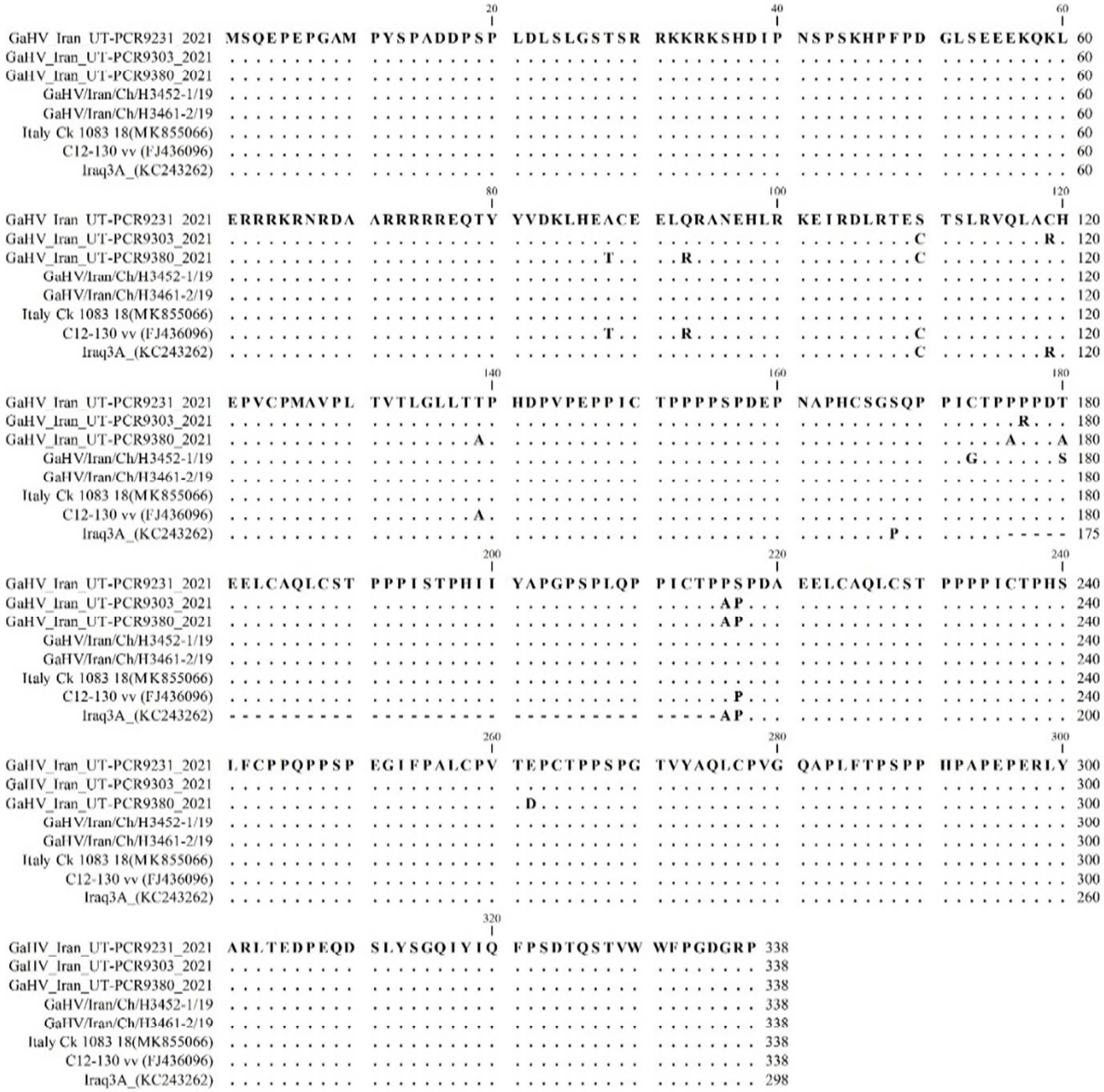

Figure 2. *Alignment of Meq protein of MDV detected from the commercial broiler flock, 2021.*

4. Discussion

Marek's disease in broiler flocks is important in two ways: carcass condemnation; and immune effects. The introduction of the first HVT vaccine in the broiler of the USA resulted in a rapid decline in the percentage of condemnations. The *in-ovo* MD vaccination for broilers is mostly used in the USA (more than 95 percent of broilers), and its use is increasing in Asia (Taiwan, Korea, Japan), Europe (mainly Spain and Italy), and South America (Argentina, Brazil) [11]. In Iran, there is no question of removing the carcass due to many brands, and the vaccine is not used in broiler flocks, and the bivalent MD vaccine (CVI-988+HVT) is just used in grandparents, breeders, and layers. A considerable increase in the rate of infection in broiler herds in this study is comparable with the group's unpublished data from the northern provinces a few years ago, showing a lower infection rate (0 to 5%). In recent years, we have also faced the occurrence of MD in layer and breeder flocks in Iran. Furthermore, this study showed that the frequency of the virus varies in different provinces of the country. As in the northern provinces, the probability of conflict is lower due to high humidity. Besides other studies, this study can complete some of the valuable information on

the epidemiology of this disease in Iran. Farhoodi *et al.* (2007) studied MD in broiler flocks in three provinces of Iran. Twenty-seven percent (27%) showed positive PCR reactions with gross and microscopic lesions, including red leg, swollen feather follicles, and lymphoblast accumulation in the dermis, while three percent (3%) showed positive PCR assay without the presence of gross or histopathology lesions [12]. Hablolvarid *et al.* (2011) estimated the incidence of MD in broiler flocks of some major regions of chicken rearing in Tehran province. Gross and microscopic examinations of chickens in the four mentioned regions showed that 24 out of 80 flocks (30%) had been infected with different disease forms. This result indicated that MD has a high incidence in broiler flocks of Tehran province. The incidence of cutaneous, visceral, and mixed cutaneous and visceral forms in these regions (four regions) was determined as 16.2%, 3.8%, and 10%, respectively [13]. Doosti *et al*. (2011) carried out a molecular study to detect MDV in southwest Iran. From 280 samples, 62.86% were positive on PCR analysis [14].

The results of this study revealed that various strains of the virus are present in broiler flocks in Iran. Although their pathotypes have not been determined *in vitro*. It has been reported that MDV1 strains with few copies of the proline-rich repeat region of *Meq* tended to be highly virulent strains, and the numbers of the proline motifs gradually lessened along with the increased virulence level [15]. Thus the range of two to three pppp repeats of *Meq* sequence strains in this study could be probable evidence of virulence. Based on sequencing and phylogenetic analysis of the *Meq* gene from layers of Iran, Ghalyanchi *et al*. (2022) put GaHV-2 isolates into two separate clades regarding molecular features. One clade containing strains was closely related to Italian, Indian, and Hungarian virulent (v) isolates, and the other clade was related to American very virulent plus (vv+) isolates [10].

5. Conclusion

On the one hand, broiler flocks, as an epidemiological reservoir involved in the transmission and genetic modification of the virus; directly and indirectly, can cause problems. On the other hand, MD vaccines have probably driven MDV to greater virulence, because they do not induce sterilizing immunity. Vaccinated chickens constitute ecosystems where vaccine and virulent MDV coexist, and mutations and recombination readily occur [11]. Therefore, considering the non-use of the vaccine in broiler flocks, the question of whether the vaccine should be used in Iran is a crucial decision. Two generations of Marek's disease vaccines have shown reduced efficacy over the last half-century due to the virus's evolution worldwide. Understanding which pathotypes and where they are present, may give insight into whether continuous reductions in efficacy are likely. Given the limited evidence of this study and the other studies [10-14], more epidemiological and virological studies; particularly to confirm the pathotypes of the strains; are needed to show the beneficial effects of vaccine in broiler flocks in Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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