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Research article

Genotypic characterization of Marek's disease virus from clinically suspected layer in Basrah, South of Iraq

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Abstract

A reemerging viral disease known as Marek's disease affects chickens and has a devastating financial impact on the global poultry sector. The emergence of pathogenic strains must be continuously monitored using molecular approaches of Marek's disease and to adevise to use appropriate vaccination program with implement bio-security. This study was continued from May 2021 to August 2021. Clinical symptoms and postmortem lesions were used to identify MD, and PCR testing for the Meq gene provided further confirmation. The meq gene's phylogenetic analysis was also performed, and such test revealed clustering of the our Iraqi field strains with Euroasian cluster, where the genetic characteristics of the present study MD strains were similar to those of Chinese and European strains.

Keywords: Clinical finding, Mareks disease, Partial sequence, PCR, Phylogenetic analyses

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INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens induced by an alphaherpesvirus, Marek's disease virus (MDV), initially described by Joseph Marek 1907. The genus Mardivirus that was first isolated in 1968 (Gall et al., 2018). MD is characterized by peripheral nervous system alterations as well as diffuse or nodular tumors in the viscera, muscle, and skin (Witter et al., 2010). There are four stages in the pathophysiology of an MDV infection. A first phase that lasts between three and six days post infection. Bursa derived (B) lymphocytes are the main target cells for infection. The second phase involves infection of thymus-derived (T) cells that are activated CD4+. In the second phase, viral replication normally slows down and a latent infection develops in activated CD4+ T cells between 5 and 10 days post inoculation. Between 14 and 21days post infection, the third phase is distinguished by the reactivation of MDV replication and the infection of feather follicle epithelium (FFE) cells (Boodhoo et al., 2016). Virus is shed from the chicken in dried FFE cells after the third phase, and depending on the genetic vulnerability of the chickens and the virulence of the virus strain, clinical MD signs and lymphomas may appear. Direct or indirect contact with hens via the aerial pathway is an easy way to spread MDV (Jarosinski et al., 2007). MD is brought on by breathing contaminated chicken house dust and dander, where the virus can linger for a very long time (Adedeji et al., 2019). Infected birds continue to spread the virus into the surroundings of the poultry house for the rest of their lives. FFE creates completely contagious viruses (Sullivan et al., 2005). The primary source of environmental contamination and chicken illness is due to the cells found in feathers and dander. Contaminated chicken house dust is infectious for years at 4°C and for at least several months at 20°C and 25°C. Although there is no vertical transmission (transmission from chicken to egg), there is horizontal transmission (transmission from bird to bird after hatching) (Lee et al., 2013). The MDV genome codes for a number of distinctive proteins, some of which have been linked to the virus's oncogenicity. The most well investigated MDV gene, Meq, produces a protein that has a high degree of similarity to the jun/-fos family of transcriptional factors. All MDV converted cells consistently express Meq, indicating that it may be crucial for transformation (Arulmozhi et al., 2011). MDV is a cell-associated herpes virus with lymphotropic properties similar to gammaherpes viruses. However, its molecular structure and genomic organization are similar to alphaherpes viruses. The International Committee on Taxonomy of Viruses (ICTV) recently classified all MDV serotypes under the umbrella of the Mardivirus genus. Gallid alphaherpes virus 2 (serotype 1), Gallid alphaherpes virus 3 (serotype 2), and Meleagrid alphaherpes virus 1 are members of the genus Mardivirus, which was previously divided into three serotypes (herpesvirus of turkey [HVT], serotype 3). The prototype virus for this family of avian viruses is serotype 1 MDV, and unless stated otherwise, MDV refers to serotype 1 virus. Serotype 1 strains are further broken down into pathotypes based on their virulence, which are frequently referred to as mild (m)MDV, virulent (v)MDV, very virulent (vv)MDV, and very virulent plus (vv+) MDV strains. Gallid herpesvirus 2, a non-cancerous virus 2 isolated from chickens and HVT belonging to genus Mardi virus (Witter et al., 2010). In Iraq, especially in Basrah province the laying hen's industry consider as new experience, because the market was dependent on eggs imported from

neighboring countries. This disease is one of the problems in world poultry industries, also there was no previous official scientific report related to MD in layer chickens in Basrah. Therefore, the our investigation was conducted to identify the molecular characterizations of such virus and determine the circulating strains of studied farm.

MATERIALS AND METHODS

Sample collection and processing

At the start of May 2021, in the Department of Pathology and poultry diseases College of veterinary medicine, university of Basrah, we received some information from one owner who has a small poultry house for egg production, and this information included, some birds suffering from nervous signs. After that our team a poultry diseases specialist visited and examed birds in this poultry house. Our decision was a primary clinical diagnosis as MD, and because there was no previous official report in Basrah from layers chickens, as well as, this type of chicken breeding and production is a very new experience in Basra, because the market was dependent on eggs imported from neighboring countries. immediately put a plan for monitoring this case.

The present study focused on laying hens house, this poultry house was not officially, designed and built out of the sanitary condition guidelines of poultry house requirements which are located in Basrah, Iraq. The poultry house included a one thousand hens of two types of chickens, Brown and White Lohmann at different ages and unequal numbers. However, the age was from 24-60 weeks. The duration of this study was continued from May 2021 to August 2021. The samples were taken from 40 chickens clinically suspected and diagnosed as MD. The chickens were collected and transported to the Department of Veterinary Pathology and Poultry Diseases at the Faculty of Veterinary Medicine, University of Basrah-Iraq for recording all clinical signs in detail and postmortem examination.Chickens that show tentative as MD were euthanized and examined individually to record a postmortem change. Nine samples were collected from the sciatic nerve, spleen, and intestine and stored at -80 for molecular work. The postmortem change planning was processed according to (OIE, 2010). The current study was performed under the permission of the ethical committee in the Faculty of Veterinary Medicine, University of Basrah (Ref. No. 74/2021). Our data was calculated as percentage.

DNA Extraction and Polymerase chain reaction (PCR)

DNA was extracted from all samples from tissues of laying hens (sciatic nerve, spleen, and intestine) that were suspected of MDV according to the gSYNCTM DNA Extraction kit quick protocol (Geneaid, Korea). Using NanoDrop, the amount of purified DNA was calculated. Prior to use, the chromosomal DNA samples were kept at -20°C. By using PCR, all recovered DNA samples were verified. The forward primer (5′-GGAT CGCCCACCACGATTACTACC-3′) and reverse primer (5′-ACTGCCTCACACAACCTCATCTCC-3′) were used in the PCR procedure to identify the Meq gen of MDV (roughly 319 bp) (Demeke et al., 2017). PCR techniques were carried out with the AccuPower® PCR PreMix Set (Bioneer, South Korea), which included 20 µL. The thermo cycler: a program was a pre-denaturation phase at 95 °C for 2 min, then 35 cycles of denaturation at 95 °C for 60 sec, annealing at 40 °C for 60 sec, and extension at 72 °C for 90

sec, plus a final extension step at 72 °C for 7 min. A 1.5% (w/v) TAE agarose gel with the proper number of wells filled with DNA samples and stained with 1 to 3 l ethidium bromide dye was used. For 50 minutes, the agarose gel was conducted at 85 V. The DNA contained in the agarose gel was seen using a UV transilluminator.

Sequence and Bioinformatics analysis of the meq gene

The Promega DNA Cleanup kit was used to cut from the gel and purify the PCR products produced in the manner previously described. Using either the forward or reverse primer for the Meq gene of MDV, nucleotide sequencing of the purified PCR products was carried out in both orientations. A publicly traded biotechnology firm in South Korea carried out the sequencing (Macrogen). The GenBank database sequences (Table 1) used in the present study were aligned and compared with that of our studied sequences.

Table 1 Represent scientific name and accession numbers of reference strains of gene bank which uses in the present study

No	Scientific name	Accession No.
1	*G. alphaherpesvirus* 2	MG432697.1
2	*G. alphaherpesvirus* 2	KU744561.1
3	*G. alphaherpesvirus* 2	KU744560.1
4	*G. alphaherpesvirus* 2	KU744559.1
5	*G. alphaherpesvirus* 2	KU744557.1
6	*G. alphaherpesvirus* 2	KU744556.1
7	*G. alphaherpesvirus* 2	JX844666.1
8	*G. alphaherpesvirus* 2	AF147806.2
9	*G. alphaherpesvirus* 2	JQ809691.1
10	*G. alphaherpesvirus* 2	JQ806362.1
11	*G. alphaherpesvirus* 2	JQ806361.1
12	*G. alphaherpesvirus* 2	MF431495.1
13	*G. alphaherpesvirus* 2	KU744558.1
14	*G. alphaherpesvirus* 2	KT833852.1
15	*G. alphaherpesvirus* 2	KT833851.1
16	*G. alphaherpesvirus* 2	MZ054335.1
17	*G. alphaherpesvirus* 2	MT872313.1
18	*G. alphaherpesvirus* 2	MT797631.1
19	*G. alphaherpesvirus* 2	MT797630.1
20	*G. alphaherpesvirus* 2	MT797629.1
21	*G. alphaherpesvirus* 2	JQ809692.1
22	*G. alphaherpesvirus* 2	EF523390.1
23	*G. alphaherpesvirus* 2	AY510475.1
24	*G. alphaherpesvirus* 2	AF243438.1
25	*G. alphaherpesvirus* 2	JQ836662.1
26	*G. alphaherpesvirus* 2	JQ820250.1

RESULTS

Clinical history and gross lesions

During the study period, the infected of poultry house included a one thousand hens, a total of 40 laying hens shows severe clinical signs as MD. This clinical result represent 4% of total hens. Most of the affected birds show identical typically signs such as paralysis or paresis, which starting with one leg, and the paralysis progressed to involve other parts of the body including one wing appearing lower than the other represented as a fan shape, leading to difficulties in standing, walking, and even complete immobility. Then these affected birds were appeared, depressed, inactive, significant weight loss and show a lack of energy, as shown in Figure 1.

Figure 1 Classical form of MD,(A); Unilateral paralysis of the legs and wings, (B) Complete spastic paralysis as fan shape

In postmortem examinations of 40 clinically affected layer hens, all these chicken have lesions, whereas 9 of 40 birds were exhibited with more clear and characteristic findings in visceral organs. Affected organs containing tumors ranging in size and appearance, also discoloration from grayish-to-yellowish, indicating the presence of tumors or infiltration by the virus. Grossly visible tumors or nodules in organs include the spleen, intestine, heart, and sciatic nerve represented in Figure 2. The spleens in some affected chickens were five times larger than the normal size that referred as splenomegaly depicted Figure 2A. Intestinal changes, including enlargement, thickening, and nodular tumors observed as raised areas within the intestinal tissue as in Figure 2B. The affected heart appeared tumors within the heart muscle and congested, which refers to the pooling of blood in the chambers or blood vessels. The congestion may manifest as a reddish or bluish discoloration in Figure 2C. In addition to enlargement, the affected nerves show signs of thickening, congestion, and edematous, Figure 2D.

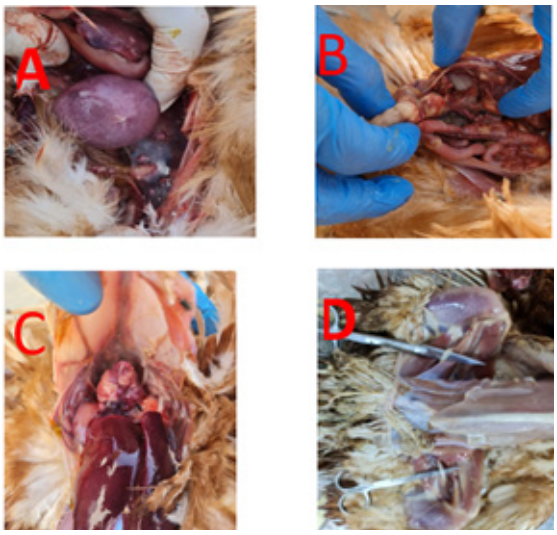

Figure 2 Macroscopic lesions of infected chickens (A) Splenomegaly with nodular tumor. (B) Intestinal changes, including enlargement, thickening, and nodular tumors. (C) Heart appeared tumors within the heart muscle and is congested. (D) Sciatic nerve congestion, enlargement, and edematous.

Detection of DNA by PCR

To confirm the precise existence of an approximately 319bp DNA product for the meg gene of MDV, PCR amplification for the MDV was carried out using the extracted DNA. The good examples we provide are shown in Figure 3.

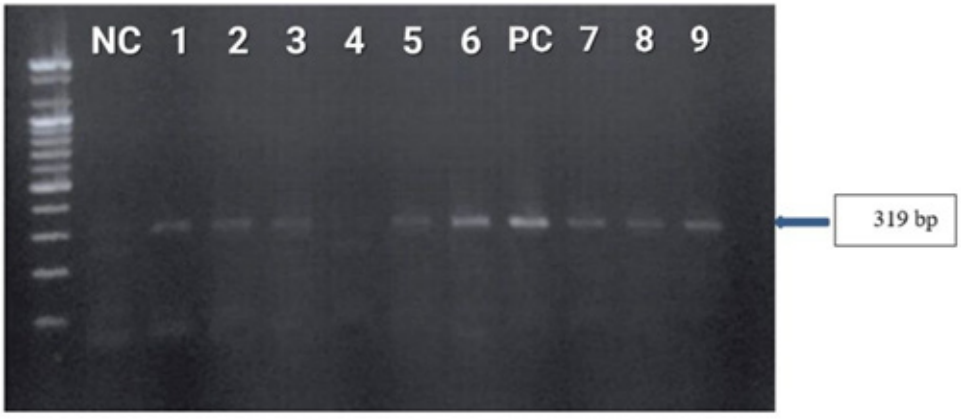

Figure 3 MDV meq gene Amplification, approximately 319bp , Lane1, 2,3,4,5,6,7,8and 9 studied samples, (NC)Negative Control , (PC) positive Control.

Sequence and Bioinformatics analysis of the meq gene

As many examined laying hens' chickens that were suspected of having MDV had samples of GaHV -2 strains, which were discovered by PCR assay. Our studied strains' with meq genes had a length of about 319 bp. The sequences of present study were aligned and compared with that of reference strain as illustrated in phylogenetic tree Figure 4. The determined Meq protein's molecular properties in comparison to strains which are presented in Table 1. A phylogenetic analysis showing relationship between *G. alphaherpesvirus* haplotypes. Each Colour represents a strain as show in Figure 4. A pairwise alignment show that nucleotide sequences of *G. alphaherpesvirus* (MD_Basra_IQ1) are translated into amino acids then compared with a reference sample of *Gallid alphaherpesvirus* 2 (MG432697.1). An identity was 99% with a reference satrain. Boxed sequences indicate nonsynonymous substitutions in amino acid sequences as we show in Figure 5. A pairwise alignment shows that nucleotide sequences of *G. alphaherpesvirus* (MD_Basra_IQ2) are translated into amino acids then compared with a reference sample of *Gallid alphaherpesvirus* 2 (MG432697.1). An identity was 82% with a reference sample. Colored amino acid sequences indicate that the amino acid in the reference sample is replaced with a nonsynonymous amino acid in *G. alphaherpesvirus* (MD_Basra_IQ2) As in Figure 6.

A pairwise alignment shows that nucleotide sequences of *G. alphaherpesvirus* (MD_Basra_IQ3) are translated into amino acids then compared with a reference sample of *Gallid alphaherpesvirus* 2 (MG432697.1). An identity was 97% with a reference sample. Boxed sequences indicate nonsynonymous substitutions in amino acid sequences.as in Figure 7.

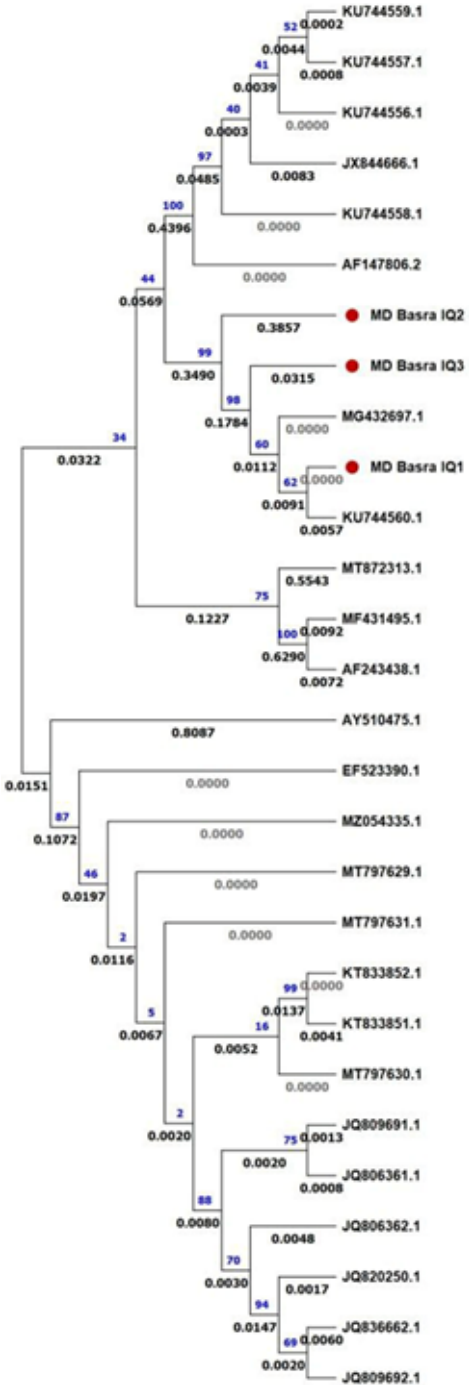

Figure 4 Phylogentic relationship of Gallid alphaherpesvirus 2 (representative strains) with the reference strains from different countries based on meq gene sequence. Phylogentic tree was constructed by neighbor –joining method with mega 11 software, by 1000 bootstrap replication. The, MD/Basra/IQ1, MD/Basra/ IQ2 , and MD/Basra/ IQ3- our studied strains.

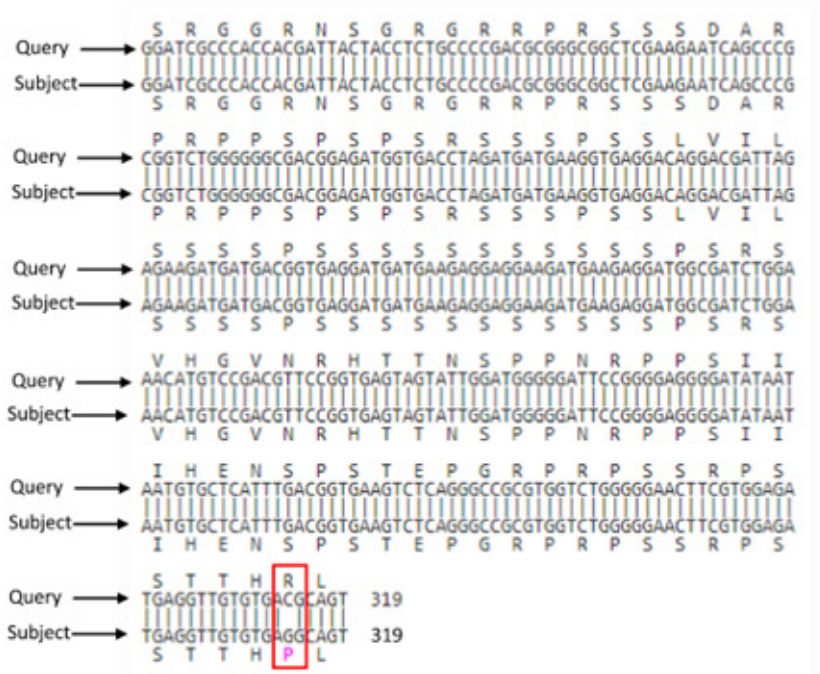

Figure 5 A pairwise alignment shows that nucleotide sequences of G. *alphaherpesvirus* (MD_Basra_IQ1) are translated into amino acids then compared with a reference sample of *Gallid alphaherpesvirus* 2 (MG432697.1). An identity was 99% with a reference sample. Boxed sequences indicate nonsynonymous substitutions in amino acid sequences.

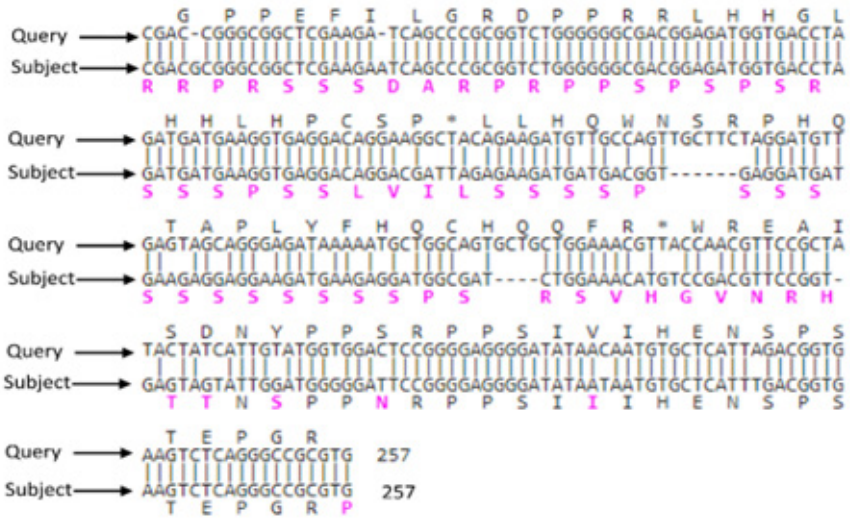

Figure 6 A pairwise alignment shows that nucleotide sequences of G. *alphaherpesvirus* (MD_Basra_IQ2) are translated into amino acids then compared with a reference sample of *Gallid alphaherpesvirus* 2 (MG432697.1). An identity was 82% with a reference sample. Colored amino acid sequences indicate that the amino acid in the reference sample is replaced with a nonsynonymous amino acid in *G. alphaherpesvirus* (MD_Basra_IQ2).

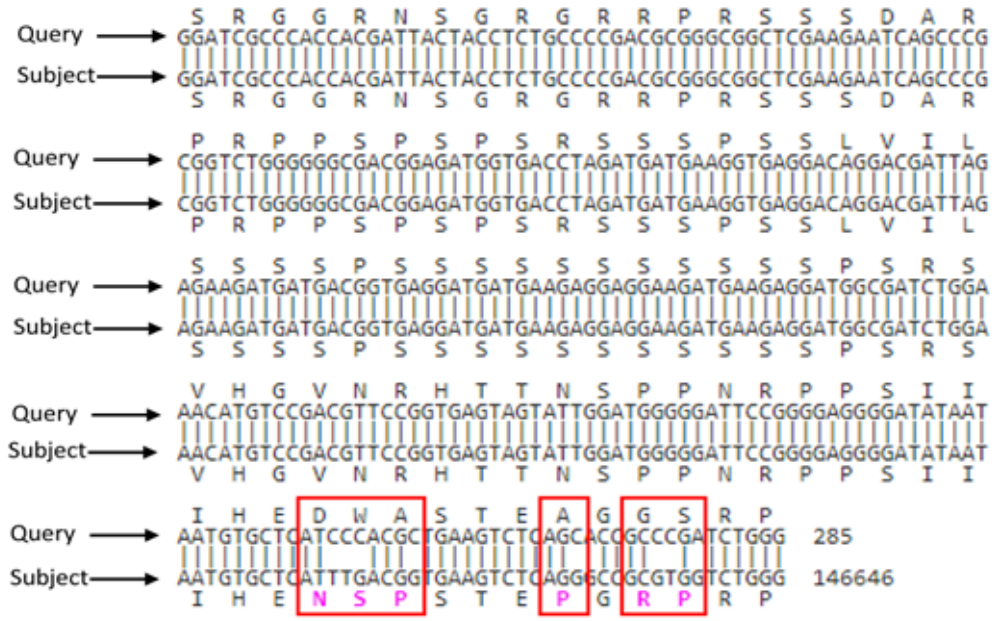

Figure 7 A pairwise alignment shows that nucleotide sequences of G. *alphaherpesvirus* (MD_Basra_IQ3) are translated into amino acids then compared with a reference sample of *Gallid alphaherpesvirus* 2 (MG432697.1). An identity was 97% with a reference sample. Boxed sequences indicate nonsynonymous substitutions in amino acid sequences.

DISCUSSION

MD is a significant viral illness that affects poultry and is linked to immunosuppression, paralysis, and rapidly developing lymphoid tumors. due to the incredibly infectious MDV. Infected chicken house dust and dander, where the virus can persist infectious for a long time, is that causes MD to begin (Dunn and Gimeno, 2013). Affected birds are contagious for life and constantly shed the virus, contaminating the environment in poultry houses. (Nair, 2018).

In the present study, we describe forty cases of laying hens 24-60 weeks of age that were suffered from clinical signs loss in appetite, depression, weakness, weight loss, ataxia and paralysis one leg stretched forward and the other back Paralysis of the legs, wings, (fan shape) this results in agreement with results of (Fenner et al., 2013; OIE, 2010). Whereas this clinical result which mentioned above represent 4% of total hens, this result related to (Lounas et al., 2021), who reported that the mortality rate varied from 4% to 10% in broiler breeder.

Also (Trang et al., 2022) found prevalence of MDV in backyard chickens in Vinh Thanh (3.57%) and Thoi Lai districts (5.43%) in vietnam.

The clinical manifestations associated with tumors in visceral organs due to viral infection with multiple lymphoid and neurological degeneration leading to paralysis and nervous symptoms. Lesions in various organs as spleen, intestine and heart was firm consistency, smooth surfaces, grey in color as well as nodular like tumor with varying different size, as well as enlargement and edematous appearance in sciatic nerve. These results of postmortem changes

that were considered as characteristic postmortem for MDV in this study which agreement with (Abdallah et al., 2018; Abd–Ellatieff et al., 2018). The variation of gross lesions in organs but not in all birds recorded similar lesion exactly which were suggestive different immune state, breed of birds and strain of virus (some virus strains are more virulent than others).

The positive PCR samples showed solid bands in gel electrophoresis but, there was one sample that failed to exhibit band, perhaps due to the presence the PCR inhibitors or low viral load in this one sample.

For the first time, the present study provides molecular insights into the GaHV-2 strains currently circulating in poultry industry, expanding the knowledge on MD in the Basra/Iraq. Our strains, detected in chickens exhibiting typical MD clinical signs and gross lesions, were confirmed molecularly by meq gene sequences. In the current research, we analyzed the partial meq gene sequence of MD/Basra/ IQ1-IQ3 strains in 2022 to compare the genetic traits of MDV strains circulating in Basra province/Iraq.

The distinguished diversity of the meq gene has been regarded to be related with virulence of MDV strains (Shamblin et al., 2004; Padhi and Parcells, 2016). Meq gene is thought to be associated with the evolution of MDV virulence, and non-synonymous mutations. (Trimpert et al., 2017).

The nucleotides similarity of the present Iraqi MD strains were related to Chinese alphaherpesvirus 2 strain HNGS101 and euro alphaherpesvirus 2 strain EU-1, which clustering with Euro Chinese strains; that may be attributed to importing some types of chicken especially laying hens and pet chickens from China and some of the European countries.

The phylogenetic part of our study include; nucleotide similarity of the local present strains ranged from 98-100% for all three local strains due to point mutation at various positions. Although all strains are from the same area, the variation may be attributed to mutation capacity of the field strains, vaccines and vaccine immunity could be other reasons that attributed for emergence of virulent MD strains. While in the amino acid sequences there are non-synonymous substitutions; GaHV-2 is a DNA virus, but its Meq gene exhibits a high mutation rate of about 10-4 mutations annually, similar to RNA viruses (Padhi and Parcells, 2016). The present result were related to (Wajid et al, 2013) who mentioned Iraqi meq sequences also contained single-nucleotide polymorphisms, resulting in some differences in the amino acid sequence. Meq genes encoded two repeats of four-proline sequences, and the published negative association between four-proline repeat and MDV virulence suggests that the Iraqi MDV strains are likely to be highly virulent. The Iraqi MDVs had a short meq gene of 897 bp because of a deletion in 123.

CONCLUSIONS

In this research, we analyzed the partial genome sequence of an Iraqi MDV strain from Basrah known as MD/Basra/IQ1-IQ3 and looked at its genomic features. These populations' genomic characteristics tended to resemble those of Chinese and European strains.

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AUTHOR CONTRIBUTIONS

Harith Abdulla Najem: contributed to the design of the research and field diagnosis of the disease.
Isam Azeez Khaleefah: molecular detection.
Waleed Majeed Almayahi: genetic analysis of the results and to the writing of the manuscript.

CONFLICT OF INTEREST

There are no competing interests of the writers.

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