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المجلة العراقية لبحوث السوق
وحماية المستهلك

MOLECULAR DETECTION OF MAREK'S DISEASE IN IRAQI POULTRY FARMS

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ABSTRACT

Marek's disease is a highly contagious, immunosuppressive, lymphotropic, and economically effective illness of avian species, mainly chickens, caused by a herpes virus known as Gallid herpesvirus 2 of the subfamily Alphaherpesvirinae. In this study, the prevalence of Marek's disease in different Iraqi governorates was investigated using highly specific and sensitive primers. The results revealed that twelve farms out of the total 50 tested were positive for Marek's virus representing 24%. The highest rate of infected farms was recorded in Baghdad with 4 farms (23.5%) followed by Diyala, Wasit, and Karbala at infection rates of 2(25%), 3(27.3%), and 3(100%) farms respectively. In addition, the study showed that the infection was recorded in two time periods during the study duration: the first one from February through April, and the second one from August through November. The affected bird's age was also analyzed, with an inconclusive rate. In conclusion, this study provides molecular evidence of Marek's disease circulation within Iraqi poultry farms even under a vaccine control strategy.

Keywords: Marek's Disease virus, Real-time PCR, Iraq.

التحري الجزيئي عن فايروس الميرك في مزارع الدواجن العراقية

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الخلاصة

يعد مرض الميرك من الامراض السرطانية المعدية عالية الخمج والمسببة للإخماد المناعي والخسائر الاقتصادية الكبيرة حيث يصيب الدواجن بصورة رئيسية. يسبب المرض فايروس من نوع الهربس Herpesvirus يعرف باسم 2 Gallid herpesvirus والذي يعود الى ما تحت العائلة Alphaherpesvirinae. تم في هذه الدراسة التحري عن مدى انتشار مرض الميرك في حقول الدواجن في العديد من محافظات العراق من خلال استخدام بادئات جزيئية عالية التخصص والحساسية. كشفت النتائج وجود 12 حقلاً مصاباً بالفايروس من مجموع 50 حقلاً تم فحصها خلال الدراسة وبما يمثل 24%. كان اعلى مستوى للحقول المصابة في محافظة بغداد وبواقع 4 حقول تمثل نسبة 23.5% من مجموع الحقول المفحوصة للمحافظة، تلتها محافظات واسط وكربلاء وديالى بواقع إصابة 3(27.3%)، 3(100%)، و2 (25%) على التوالي. بالإضافة الى ذلك، فقد سجلت الدراسة حدوث الإصابات على مدى فترتين زمنيتين خلال مدة التحري، الأولى من شهر شباط الى شهر نيسان والثانية من شهر اب الى شهر تشرين الثاني، ولم تقدم بيانات اعمار الطيور المصابة نتائج قطعية عن العمر الأمثل للإصابة. اثبتت هذه الدراسة وجود إصابات حقلية بفايروس الميرك في مزارع الدواجن العراقية رغم استخدام التلقيحات الوقائية كأسلوب للسيطرة على المرض.

الكلمات المفتاحية: مرض الميرك، تفاعل انزيم البلمرة،العراق.

INTRODUCTION

The poultry industry is considered one of the important sources of food security around the world **(Daghir *et al*., 2020)**. The intensive increase in this industry during the last century faced many challenges, especially avian pathogens like Avian influenza **(Mahmood & Allawe, 2020, Allawe & Hidayat, 2022)**, Newcastle disease virus **(AL-Zuhariy *et al*., 2017)**, infectious bronchitis virus **(Ali & Allawe, 2023)**, as well as Marek's disease infection **(Alkubaisy & Hameed, 2023)**. Marek's disease is a highly contagious, lymphotropic disease of chickens mainly, caused by a virus known as Gallid herpesvirus 2 belongs to the Alphaherpesvirinae of the family Herpesviridae **(WOAH, 2023)**.

The annual economic losses worldwide wide were estimated at 1-2 billion US dollars with an expected possible increase due to vaccine failure and the evolution of higher virulent virus strains **(Dima & Girma 2021)**. Losses appear with high mortality rates, emaciation, and loss of condition, losses to the eggs industry, gross visceral lesions that lead to the discarding of carcasses, and additional costs funds for research and vaccine development **(Rozins *et al*., 2019; Bertzbach *et al*., 2020; Hassan & Abdul-Careem, 2020; Dejyong *et al*., 2023)**. The infection is initiated through airborne viruses with dust and feather follicle epithelium that are inhaled into a bird's lung where a new viral cycle starts. **(Boodhoo *et al*., 2016)**. The disease is characterized by a wide range of clinical signs, involving lymphoma in several visceral organs and neural pathogenesis that causes paralysis of legs and wings as well as brain edema **(Witter, 1997; WOAH, 2023)**.

During its life cycle, MDV showed a complex, multi-stage pathogenic manifestation, during which, multiple immune cells are involved. The pathogenesis starts with the initial infection stage that takes place in chicken lungs and involves the association of macrophages, dendritic cells, and some B-lymphocytes. This stage ensures the establishment of infection, and virus dissemination to the bursa of Fabricius, thymus, and spleen, where T lymphocytes, especially CD4+ subsets are infected. Infection of T-lymphocytes is essential to start the second pathological stage: Latency, which starts about 7-10 days after infection. Those latently infected T cells are transmitting the virus to different body organs including the feather follicle epithelium where the third stage of reactivation starts. After 28 days of infection, a proportion of latently infected t cells is transformed into lymphoma and invade many internal organs and tissue, representing the lymphoma form of the disease **(Bertzbach *et al*., 2020; Worku, 2022)**.

Marek's disease is controlled mainly by vaccination side by side with biosafety management, a combination that ensures the minimum passive impact of the disease on poultry flocks. Vaccines, at proper administration and under accurate barn management will ensure controlling the mortality and acute clinical signs as well as prevent lymphoma, although these vaccines still permit bird infection and shedding virus to the environment **(WOAH, 2023)**.

Although anatomy or post mortem regarded as the main technique for the initial diagnosis of MDV through the main gross pathological characteristics, other laboratory methods are highly recommended like histopathology, radial agar gel immune diffusion test, virus isolation, and PCR with variable sensitivity and specificity for each technique **(Krol *et***

***al.*, 2009; WOAH, 2023).** In Iraq, the continuous application of vaccination has played a great role in decreasing the clinical cases of MDV. In this work, real-time PCR was selected to survey the infectious rates of MDV and connect them with some epidemiological parameters for more disease understanding.

MATERIALS AND METHODS

Samples collection

This study targeted a total of 50 layer farms during 2022-2023 from ten Iraqi provinces (Table 1). The criteria for farm selection depend mainly on clinical suspection of MDV during postmortem like enlarged of the liver, spleen, and sciatic nerve. Samples of the liver, spleen, and kidney, as well as enlargement feather follicles, were collected. Samples were prepared for testing by chopping them into very small pieces using scissors and forceps, centrifuging for 10 minutes at 2500 rpm, and collecting the supernatant for the subsequent test procedure.

Table (1): Representing sample number, origin, and time of collection for this study.

	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Baghdad	1	/	5	5	2	2	/	/	/	2	/	/
Diyala	/	1	3	/	1	1	/	/	/	2	/	/
Wasit	1	3	1	2	/	/	/	/	1	3	/	/
Karbala	/	/	/	/	/	/	/	1	1	/	1	/
Al najaf	1	/	/	/	/	/	/	1	/	/	/	/
Diwanyia	3	/	/	/	/	1	/	/	/	/	/	/
Thi-Qar	/	/	1	/	/	/	/	/	/	/	/	/
Basrah	/	/	/	1	/	/	/	/	/	/	/	/
Al anbar	/	/	/	/	1	/	/	/	/	/	/	/
Kirkuk	/	/	/	/	/	2	/	/	/	/	/	/

Viral Nucleic acid extraction

Viral DNA was extracted from suspected samples using the kit provided by QIAGEN® extraction kit according to the manufacturer's instructions. The process briefly involved mixing 0.2 ml of sample with an equal volume of Lysis buffer and 0.002 ml of proteinase K in an Eppendorf tube at 56C° for 10 minutes. Then 0.2 ml of absolute ethanol was added to the mixture, vortexed well, and transferred to the silica binding tube to precipitate the DNA in the silica layer. After that, two wash steps are carried out to clean and purify the DNA from any contaminated proteins or molecules, followed by an elution step to release the extracted DNA from the silica layer and rehydrate it to be ready for testing purposes. The extracted DNA was stored at -70C° until the test.

Table (2): Represent the primer-probe set used in this study

Oligo Name	Sequence 5'-3'	Reference
MDV PP38F	GAG CTA ACC GGA GAG GGA GA	**(Baigent *et al.*, 2016)**
MDV PP38R	CGC ATA CCG ACT TTC GTC AA	
MDVPP38 Pr	FAM-CTC CCA CTG TGA CAG CC-BHQ1	

Performing Real time (PCR test)

Marek's virus investigation process was achieved through testing samples by Taqman real-time PCR, using the specific primer-probe set as in Table 2. In this study, Promega master mix reaction buffed was used and prepared as follows: 2X buffer mix, 0.4 ul of enzyme mix, 2.05 ul of DEPC,1 ul of each primer,0.5 ul of fluorescent probe, and 0.05 ul of rox passive reference stain, that produces 15 ul of reaction buffer, which is completed to the final volume of 20 ul by adding 5 ul of suspected sample.

The investigation tests were carried out using the applied Biosystem Fast 7500 thermocycler under the following conditions: initial denaturation and hot start activation at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for denaturation and 60 °C for annealing and extension elapsed for 3, and 30 seconds, respectively. The fluorescence signal was read during the extension step using the FAM filter, and the results analysis was carried out according to instrument settings.

RESULTS AND DISCUSSION

After analyzing the signal obtained through The PCR amplification run (Figure 1A, 1B), with the threshold cycle at Ct35, the test showed that 12 farms out of the total 50 under investigation showed positive indicators for The MDV genome with different Ct values by real-time PCR representing 24% of the total checked flocks.

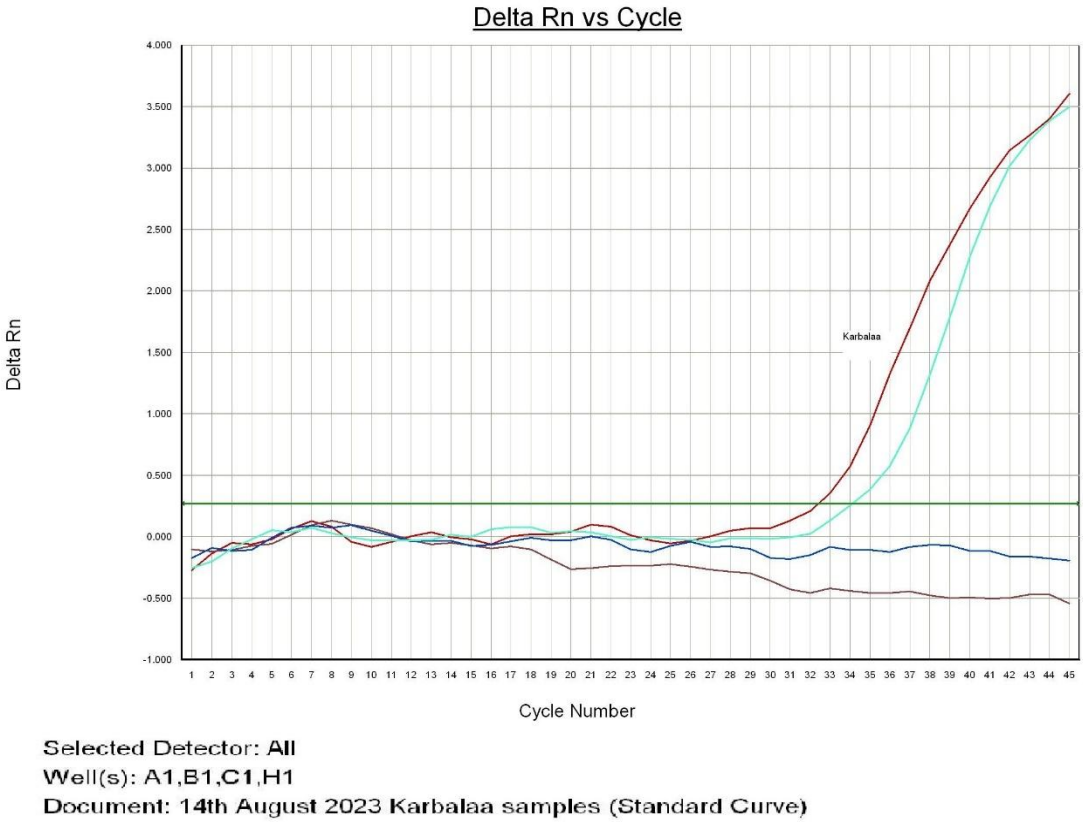

Figure (1A): Plot of Amplification curve for Karbala sample compared with the positive control

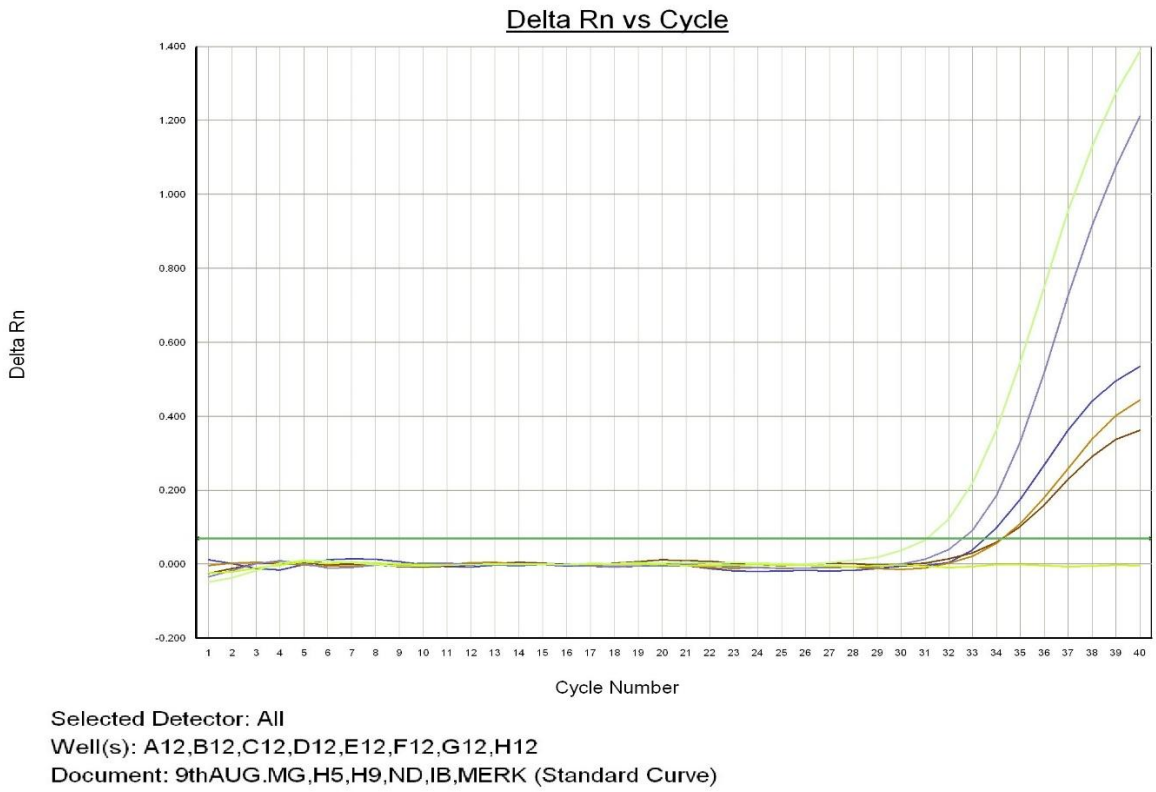

Figure (1): B Plot of the amplification curves of positive samples by real-time PCR.

Positive flocks originated from Baghdad, Diyala, Wasit, and Karbala as 4 (23.5%), 2(25%), 3 (27.3%), and 3 (100%) flock respectively (Figure 3).

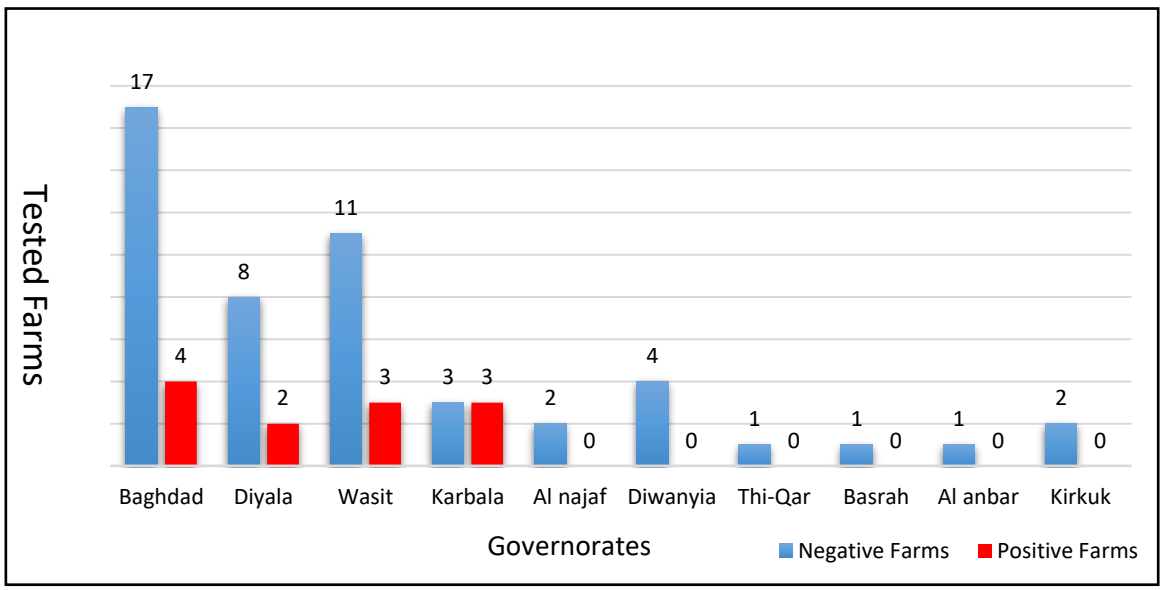

Figure (2): Represent the distribution of tested farms within the Iraqi provinces.

In addition, data based on Ct value, months, and age of flocks are present in (Figure3) and

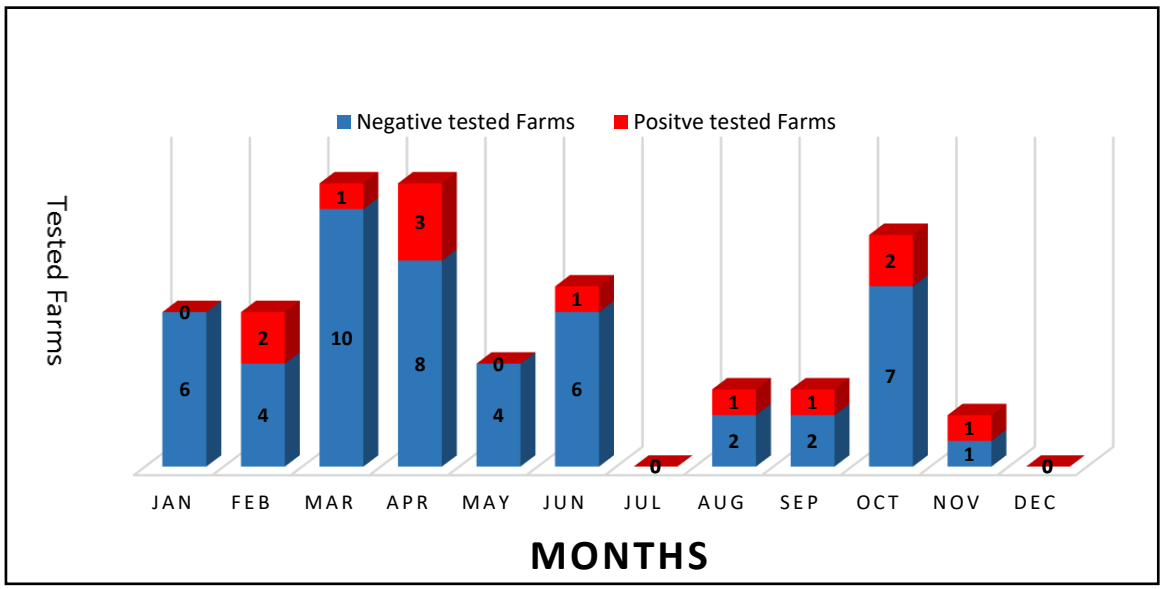

Figure (3): Represent the distribution of tested farms along the study months

Table (3): Table 1 represent location, date, age, and Ct value of positive farms.

	Province	Month	Flock age	Ct value
1	Diyala	Feb.2022	84 Days	31.1
2	Wasit	Feb.2022	112 Days	25.9
3	Baghdad	Mar.2022	161 Days	31.8
4	Wasit	Apr.2022	85 Days	33.0
5	Baghdad	Apr.2022	96 Days	34.3
6	Baghdad	Apr.2022	217 Days	33.0
7	Baghdad	Jun.2022	195 Days	26.0
8	Diyala	Oct.2022	395 Days	32.2
9	Wasit	Oct.2022	154 Days	30.0
10	Karbala	Nov.2022	150 Days	30.0
11	Karbala	Aug.2023	175 Days	33.5
12	Karbala	Sep.2023	133 Days	34.8

These results were lower than those found by **(Wajid *et al.*, 2013)** study, which detect the prevalence of MDV in southern Iraqi provinces found that total positive samples were 50% at samawa,36.8% at Thi-Qar (Nasiriya), 65.0% at Ammarah, 60% at Kut (Wasit), 36.8% at Karbala, and 45.5% at Hilla. These differences are due to using different criteria for sample selection. The positive results of this study can be analyzed at three levels: Initially, the results suggest a natural infection or reactivation of latent MDV, an indicator of active viral circulation, which agrees with the scientific base that vaccinated flocks are still susceptible to MDV circulation and shedding as mentioned by **(Bertzbach *et al.*, 2020; Islam *et al.*, 2014; Liu *et al.*, 2023; Hagag *et al.*, 2020)**. a case previously recorded by **(Zahid, 2008)** who followed up on an outbreak of MDV in a pre-vaccinated parent broiler flock in Baghdad causing a high rate of mortality within the flock. Secondly, it is very important to notice that, natural infection or reactivation of a latent MDV infection in pre-vaccinated flocks is not an outcome of exposure to the virus alone, it is the sum of synchronized factors including the virus, the susceptible host, and the environmental stress factors that enhance a successful infectious process. Many environmental stress factors play a clear role in MDV pathogenesis and the reactivation of the latent MD virus. Those factors like hunger, thirst, imbalanced ambient temperature especially overheating, bad ventilation, use of antibiotics, and exposure to mycotoxins and other avian diseases like chicken anemia virus were suggested as a predisposing factor for reactivation compatible with **(Davison & Nair, 2004; Mousa-Balabel *et al.*, 2017)** description. When applying these criteria to investigated results, positive infected flocks were recorded during (February-March-April) period in mid Iraqi provinces (Baghdad, Diyala, and Wasit). A second wave of infection was recorded in Karbala, southern Iraq, Wasit, and Diyala (August, through November). Both of these periods represent the sessions of climate change from winter to spring, and from summer to autumn, at which there are significant variations in ambient temperature between day and night, that act with improper farm management systems to form an effective stress on chickens, which match the results of **(Al-sabaawy *et al.*, 2023)**.

Additionally, bird age data were also recorded and revealed that six flocks were infected before the laying age (160 days), a sensitive time for the poultry farms as they were under the stress of increasing light intensity as well as the stress of laying eggs themselves **(Cheng *et al.*, 2021)**. This result differs slightly from the suggested typical age of infection (8-10 weeks) mentioned previously by **(Zahid, 2008)**. The environmental contamination with MDV was also recorded by **(Wajid *et al.*, 2013)**, who examined dust samples from several southern Iraqi provinces, finding that more than 50% of the testing samples were positive by molecular techniques, proving the presence of contaminated infectious particles in the environment, which supports this study results with a possible source of infection.

Thirdly, to overcome the suspicion that the results in this study are from traces of a vaccine strain and not the outcome of field infection, the selection of a highly specific primer set, that can differentiate between the field and vaccine strains utilizing a single nucleotide polymorphism (SNP) within the primer target gene PP38 **(Baigent *et al.*, 2016)**. These primers give this study results more assurance, especially since, Taqman real-time PCR utilizes double

accuracy levels (the primers and the fluorescent probe), and the analysis of results is completely mechanical using specific machine filters; there is no need for electrophoresis or human eyes to analyze results which ensures the decreasing of errors to the lowest possible levels **(Ramamurthy *et al.*, 2011)**. It is worth noting that, a real-time PCR system is very sensitive and susceptible to MDV, as it can detect minor traces of the viral genome down to 10 copies or less, which agree with **(Gall *et al.*, 2018; Wu *et al.*, 2023)** and meets the conclusion made by **(Al-Sabaawy *et al.*, 2023)**.

CONCLUSIONS

Marek's disease still represents an active threat to chicken flocks in Iraqi farms with significant economic losses at different value chains. It's valuable to think that this study gained its importance by utilizing molecular tools in active surveillance of farms within different provinces other than conventional techniques like histopathological changes or post-mortem, a process that permits the diagnosis of MDV even when the infection occurs in vaccinated flocks without distinguishing clinical features. Additionally, investigation of some epidemiological parameters like chicken age or infectious session may support the efforts to improve disease control a forward step to improve food security.

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