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Pathological and Molecular Investigation of Marek's Disease in Brown and White Lohmann Breeds in Basrah (IRAQ) (A Comparative study)

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ABSTRACT

Marek's disease (MD) remains to be one of the major problems in the production of poultry despite widespread vaccination. Recent study suggests that vaccinated flocks are breaking out, with emerging virulent strains and decreased vaccine efficacy being considered a major concern. There is limited data are available on MD prevalence in southern Iraq. This study examined a case of an outbreak in immunized commercial flocks of layers (White and Brown Lohmann) by clinical, pathological, histopathological, serological, and molecular analyses.

300 chickens were studied including 100 clinically affected White Lohmann, 100 affected Brown Lohmann and 100 control White Lohmann. Clinical symptoms consisted of asymmetric paralysis, emaciation, drooping of the wings and a mortality rate of 7.5 in Brown Lohmann and 4.2 in White Lohmann. On gross examination, hepatosplenomegaly, kidney enlargement, and swelling of the sciatic nerve were seen. Diffuse infiltration of proventriculus, lungs, heart, liver and spleen with lymphoid tumors, replacing normal tissues, was observed in 85-95 per cent of affected birds by histopathology. ELISA showed a significant decrease in ($P < 0.05$) NDV and IBV antibody in the infected birds as opposed to controls. The results of PCR confirmed that the Meq gene was positive in 100 percent of infected birds and negative in controls which had no lesions or PCR results.

The outbreak is the visceral variant of MDV in vaccinated Basra layer flocks, which indicates the existence of the extremely virulent (vv) or vv+ strains of MDV overcoming the existing vaccines. The extreme cases of immunosuppression observed emphasize the necessity of the better vaccination strategies, strict biosecurity approaches, and the constant monitoring by the means of molecular monitoring to restrain MD in large-scale poultry farms.

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Marek's disease, layer chickens, immunosuppression, Meq gene, PCR, Iraq, NDV, IBV, histopathology, vaccination failure.

1. Introduction

A highly contagious and highly disabling lymphoproliferative disease of poultry, Marek's disease (MD), is caused by the oncogenic *Gallid alpha herpesvirus 2* (GaHV-2) and develops into T-cell lymphomas, immunosuppression and neurological disorders (1, 2) Although, vaccination has greatly minimized the incidence of the MD, the war against the poultry health by the emergence of very virulent (vv) and very virulent plus (vv+) viruses still continues on a global scale (3). Layer chickens (White, Brown Lohmann) are known to be more resistant to Marek's disease than broilers, most likely because of their longer life span and enhanced adaptive immunity (4). This increased immunity has been associated with stronger humoral and cellular immunity; higher level of activation of cytotoxic T cells and γδ T cells (essential for an effective control of MDV infections) (5). Besides, transcriptomic analysis revealed that MD-resistant chicken lines have increased levels of antiviral defense pathways, including herpes simplex and influenza A, suggesting a superior innate and adaptive immunity to MDV infection (6). However, studies have shown that MDV can avoid the host immune system by inducing regulatory T cells (Tregs) that express Transforming Growth Factor Beta (TGF-β) and can cause immune suppression and enhanced tumor susceptibility (5). Besides, mutations on the viral oncogene *meq* have also been associated with increased virulence and decreased efficacy of the vaccine

which has been a subject of concern to the control of field strains (5, 6). On the continental level, economic effects of MD are mortality, poor flock performance, decreased egg production, and susceptibility to secondary infections when immunosuppression occurs (2,7, 8). The virus's immunosuppressive effect is compounded with response to other vaccines including Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) thereby increasing the overall disease challenge (1,9, 10). Data on MD in southern Iraq, particularly in the Basra region, are currently scarce, and research on the topic remains limited.

The identification of MD outbreak in vaccinated layer flocks in Basra creates serious questions to the effectiveness of existing vaccination practice and an indication of a circulation of virulent MDV strains (3, 4). The immunological mechanisms of disease resistance in layer chickens and reasons for vaccination failures are important in enhancing control strategies with local production factors (9). Therefore, the objective of this study was to investigate an outbreak of Marek's disease in vaccinated commercial layer flocks (White Lohmann and Brown Lohmann) through comprehensive clinical, pathological, histopathological, serological, and molecular characteriazation, in order to determine the virulence, genetic features- (which were measured on the basis of lesions in the organs as well as their effects on the number of organs, and the mortality rate, and specifically by gene determination (Meq gene)).and immunological implications of the circulating MDV strains.

2. Materials and Methods

Study area and flock description

The current research was done on two commercial layer farms in Basra,Iraq. In each farm, there were two flocks which were about 100,000 White Lohmann layers and 100,000 Brown Lohmann layers. Both farms were controlled and immunized in accordance with the standard commercial practices. The immunization included: Marek Disease (HVT strain): , Rispens CVI988 + HVT (Timing: On the first day. Dosage: 0.25 ml), Merial/Boehringer Ingelheim, France. Newcastle Disease Virus (NDV): LaSotta strain, provided by CEVA Animal Health, Hungary. Infectious Bronchitis Virus (IBV): H120 strain, imported by Zoetis, USA. All vaccinations were done as per the manufacture recommendations. The present investigation was initiated after clinical signs that were typical of Marek Disease were reported at the age of 175 days.

Experimental Design

In this study, the numbers of the sample were 300 chickens, which were divided into three groups including: Group 1 (n=100) White Lohmann chickens clinically affected; Group 2 (n=100) Brown Lohmann chickens clinically affected; and Group 3 (n=100) White Lohmann chickens clinically healthy which were used as controls. The control was not added for the brown breed , because there are previous study show the effect of the MD on the immunosuppression case on the brown layer breed (11), and the financial outlay of this group in this age of production . clinically affected birds were selected on the basis of the typical signs of Marek disease, i.e. asymmetric paralysis, emaciation, and wing drooping, of commercial flocks of the same age, condition of management, and vaccination regimens. A local MD-free farm was chosen using the random selection of control birds raised under similar conditions of rearing and vaccination to provide an opportunity to compare immune, histopathological, and molecular parameters.

The sample size of the study was calculated using the StatCal calculater in Epi Info® software. The initial level of calculation was based on a prevalence rate of 50 as there was no previous studies conducted in Iraq to study the prevalence of vvMD, a 99.9% level of confidence and a margin of error of 10% resulting in 271 samples. The sample size was increased to 300 birds to enhance the statistical power of the study and to account for potential data loss during sampling or laboratory analysis

Clinical examination and necropsy

Symptomatic selective birds from each affected flock were clinically examined for signs such as paralysis, emaciation and non-mobility. Birds were euthanized **in accordance with approved animal welfare guidelines** by cervical dislocation. Morphological diagnosis in postmortem suggested the presence lesions of hepatosplenomegaly and sciatic nerve enlargement, tumor-like lesions in visceral organs. Control birds were necropsied in order to demonstrate lack of macroscopic tissues that may be related to Marek's disease.

Samples collection

Both control and clinically affected birds were sampled aseptically in terms of tissue collection. The sampled organs were liver, spleen, kidneys, heart, lungs, proventriculus and sciatic nerve. One part was put in 10% neutral buffered paraformaldehyde (pH 7.4) and fixed to analyze by histopathological techniques and the other part was stored immediately at -80◦ C to conduct molecular analysis. The preservation of ultra-low temperature was done in the –80 ◦ C ultra-low temperature freezer (Model: MDF-U53V, PHCbi, Japan) supplied by PHC Corporation through the authorized distributor in Iraq (Scientific Supplies Company, Baghdad). Besides liver, spleen, kidneys, heart, lungs, proventriculus, and sciatic nerve, other aseptic tissue samples were further taken on the ovary and intestines of clinically

affected birds. Because of their significance in the occurrence of visceral lymphomatosis and systemic Marek disease infections, these tissues were expensive under the histopathological examination as well as the gross pathological examination.

There was also collection of blood sample through venipuncture at the wing vein using sterile syringes. The serum samples obtained were placed in non-anticoagulant, sterile tubes and centrifuged (3000 rpm 10 min room temperature). The separated sera were placed in a refrigeration centrifuge (Model- Sigma 3-16L, Sigma Laboratory Centrifuges, Germany) at a temperature of -20^{o} C, which would then be subjected to serological assays. External examination has been done with feather follicle swabs in order to test the existence of Marek disease virus (MDV) to possibly infected epidermal tissue. The feather follicle swabs were taken on axillary (wing) area using sterile polyester-tipped applicators which were wet with phosphate-buffered saline (PBS). All swabs were carefully twisted on the areas of the follicles and placed in 1.5 mL microtubes, each of which consisted of 500 mL of PBS, and they were placed under freezing temperatures of -80 ^{o} C until the time of DNA extraction. This method is based on the methodology given by Najam H.A., [10], which showed the practicality of feather pulp sample to detect MDV using PCR even at a low level of detection.

Histopathological examination

The tissues processed in formalin were used as per the normal preparation of paraffin embedded tissues. The tissues were thereafter dehydrated, cleared and placed into paraffin blocks. Rotary microtome (Model: Leica RM2235, Leica Biosystems, Germany) was utilized to take the tissue sections of 4,000-5,000 µm thickness to undergo a routine histopathological analysis. The sections were subsequently placed on glass superfrost plus glass slides (Thermo Fisher Scientific, USA) and stained by hematoxylin and eosin (H&E) to undertake histopathological analysis. With this technique, cellular structure, morphology of tissues and the presence or absence of pathology will be clearly observed under light microscope. The microscopic examination was carried out under light microscope (Model: CX43, Olympus Corporation, Japan) with digital imaging system along. Hematoxylin coloring of nuclei is blue, whereas cytoplasm and extracellular matrix proteins are counter stained pink by eosin, yielding the maximum contrast to identify neoplastic lymphoid infiltrates, necrosis, and tissue effacement of Marek's disease. Lesions that are typical of Marek's disease, including lymphoid infiltration, tumor formation, and nerve lesions, were assessed in slides using light microscopy. Control group tissues were also treated like base line histology (11).

Serological testing

Serum samples obtained from all birds were tested for antibodies to NDV and IBV by commercial indirect enzyme-linked immunosorbent assay (ELISA) kit (BioChek, Netherlands) following the manufacturer's instruction. Optical density (OD) values were determined at 450 nm. Antibodies titers were determined by applying the kits standard formula for Newcastle disease and Infectous Broncitous for IDVet and interpreted by using the cut-off values according to its manufacturer. Comparison of the control group with results from affected flocks was done to evaluate possible immunosuppression due to the infection(12).

Molecular detection (PCR)

Genomic DNA was extracted from frozen tissue samples (liver, sciatic nerve, and spleen) using the DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. The concentration and purity of the extracted DNA were quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). All DNA samples were stored at -20°C until further analysis. A conventional polymerase chain reaction (PCR) was performed to detect the Meq gene of Marek's Disease Virus (MDV) using a thermal cycler (Model T100, Bio-Rad, USA). Each 25 µL reaction mixture consisted of: 40 ng of DNA template, 12.5 µL of 2x PCR Master Mix(Promega, USA),and0.5 µm of each **forward (5'-ATGGGACCCCTCTTACGACC-3') and reverse (5'-GGTACCGTAGGCGTCGATGA-3') primer**. The thermal cycling protocol was as follows: initial denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 45 seconds; followed by a final extension at 72°C for 5 minutes. Each PCR run included a positive control (MDV-positive DNA) and a negative control (nuclease-free water) to ensure the validity of the amplification.

The PCR products were then separated by electrophoresis on a 1.5% agarose gel prepared in Tris-Acetate-EDTA (TAE) buffer. A 5 µL aliquot of each sample was mixed with 1 µL of 6x loading dye and loaded into the gel wells alongside a 100 bp DNA ladder (Thermo Fisher Scientific, USA). Electrophoresis was carried out at a constant voltage of 100 V for 45 minutes. The gel was stained with GelRed (Biotium, USA) and visualized under UV light using a gel documentation system (Gel Doc EZ, Bio-Rad, USA). A sample was considered positive for MDV if a clear band corresponding to the expected amplicon size of approximately 620 bp was observed(**13**).

Statistical Analysis

A statistical analysis of SPSS v26.0 (IBM Corp., USA) was undertaken. Comparison of groups' antibodies titers and PCR positivity rates were determined with one-way ANOVA, followed by Tukey's post-hoc test. A p-value < 0.05 was statistically significant. Chi squared (χ^2) test was used analyze the data of gross lesion scores (using the microscope) and mortality at P<0.05.

Ethical approval

Animal research followed all guidelines established by the Animal Care and Use Committee of the College of Veterinary Medicine at University of Baghdad. Under Protocol Number P.G.9, dated 15/2/2023, the research protocol received approval from authorities who ensure humane treatment of all participants in experiments

3. Results

The affected birds had clinical signs such as asymmetric paralysis of one or both of the legs, ataxia, and non-ability to stand with some having their wings drooping and being very emaciated. These manifestations were referred in the flocks of White Lohmann as well as Brown Lohmann. Specifically, the **Figure 1A** demonstrates an example of having a Brown Lohmann bird with asymmetry paralysis, i.e. one leg straight up and another leg straight down, whereas Figure 1B presents a cluster of White Lohmann birds lying on floor and having bilateral paralysis in legs and flattening of wings. All these clinical appearances were in line with neurological impairment that was related to Marek disease.

Organomegaly and lymphoproliferative were observed through gross pathological examination on the affected birds. Enlargement of liver was found in 90 %of both white and Brown Lohmann birds. The enlargement of spleen was found in 85 percent of White and in 90 percent of Brown Lohmann birds, and enlargement of the kidney was sighted on 80 percent and 85 percent of the animals of these groups correspondingly. The prevalence of the swelling of the Sciatic nerve in Brown Lohmann (80%) was higher as compared to that of white Lohmann (75%). In White Lohmann, 90 percent have muscle atrophy, and in Brown Lohmann, this number was 95 percent. The control group did not show gross lesions (**Table 1**). There was no significant difference ($p > 0.05$) between the flocks of White and Brown Lohmann in the frequency of entrails affected by the lesions, which means that both of the breed were presented to all the pathological effects of MDV in equal numbers. Statistically both the groups affected had lesions as zero percent in control group ($p < 0.001$). This illustrates the specificity of results to MDV infection. Such oncogenic and neurotropic behavior of MDV strain is grounded on the high rates of organomegaly and dilation of the nerve. These lesions are graphically verified in **Figure 2 (A**). swollen sciatic nerves have lost their striation; B represents irregular enlargement, C epicardial lymphoid nodules and F diffuse liver nodules that interfere with normal architecture typical of visceral lymphomatosis. Such gross and clinical evidence together with an attestation of statistical evidence gives a good indication of a visceral Marek disease caused by a strain of MDV which may be highly virulent in being capable of over-riding the vaccine-induced immunity in both layer breeds (**Figure 3**).

Table 1.Gross lesion frequency infected and Control Groups

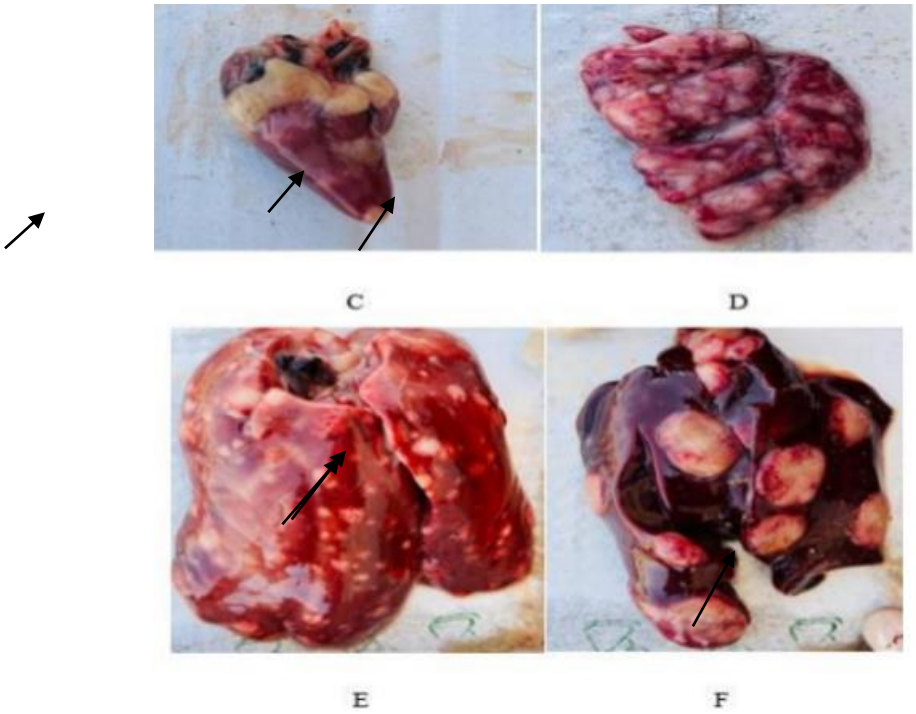
Lesion	White Lohmann (n=100)	Brown Lohmann (n=100)	Control Group (n=100)
Liver enlargement	90 (90%)	90 (90%)	0 (0%)
Spleen enlargement	85 (85%)	90 (90%)	0 (0%)
Kidney enlargement	80 (80%)	85 (85%)	0 (0%)
Sciatic nerve swelling	75 (75%)	80 (80%)	0 (0%)
Atrophic muscle	90 (90%)	95 (95%)	0 (0%)

The Chi-square test was used to determine the frequencies of gross lesions between the three groups. All the lesion types showed statistically significant difference ($p < 0.05$) between the groups of flocks infected and the control group. Nevertheless, the majority of lesions did not differ significantly ($p \geq 0.05$) between White Lohmann and Brown Lohmann except atrophic muscle where the frequency of lesions was significantly higher in Brown Lohmann ($p < 0.05$). These results were supported by post-hoc analysis by the use of Tukey HSD.



A B

Figure 1. Clinical signs of Marek's disease in commercial layer chickens. **A:** symptom of characteristic Brown Lohmann asymmetric paralysis with one leg stretched forward and another backward with wing droop. **B:** Group of White Lohmann chickens presenting like asymmetric paralysis of the legs and holding themselves down on the floor. Pest affected birds manifest as loss of coordination, inability to stand and wing drooping.



C D

E F

Figure 2. Pathological gross lesions in different organs of commercial chicken

experiencing Marek's disease. **C:** Heart with diffuse white spots on the epicardial layer resulted from lymphoid infiltration (black arrows). **D:** Kidneys with marked enlargement and mottling appearance with multifocal lymphoid nodules replacing normal parenchyma (black arrows). **E:** Lungs which presented diffuse whitish nodular lesions suggestive of neoplastic lymphoid infiltration (black arrows) compromising respiratory function. **F:** Liver with multiple large abdominal whitish nodules (black arrow) scattered within the hepatic tissue, representing pathologically normal architecture as with advanced visceral lymphoma.

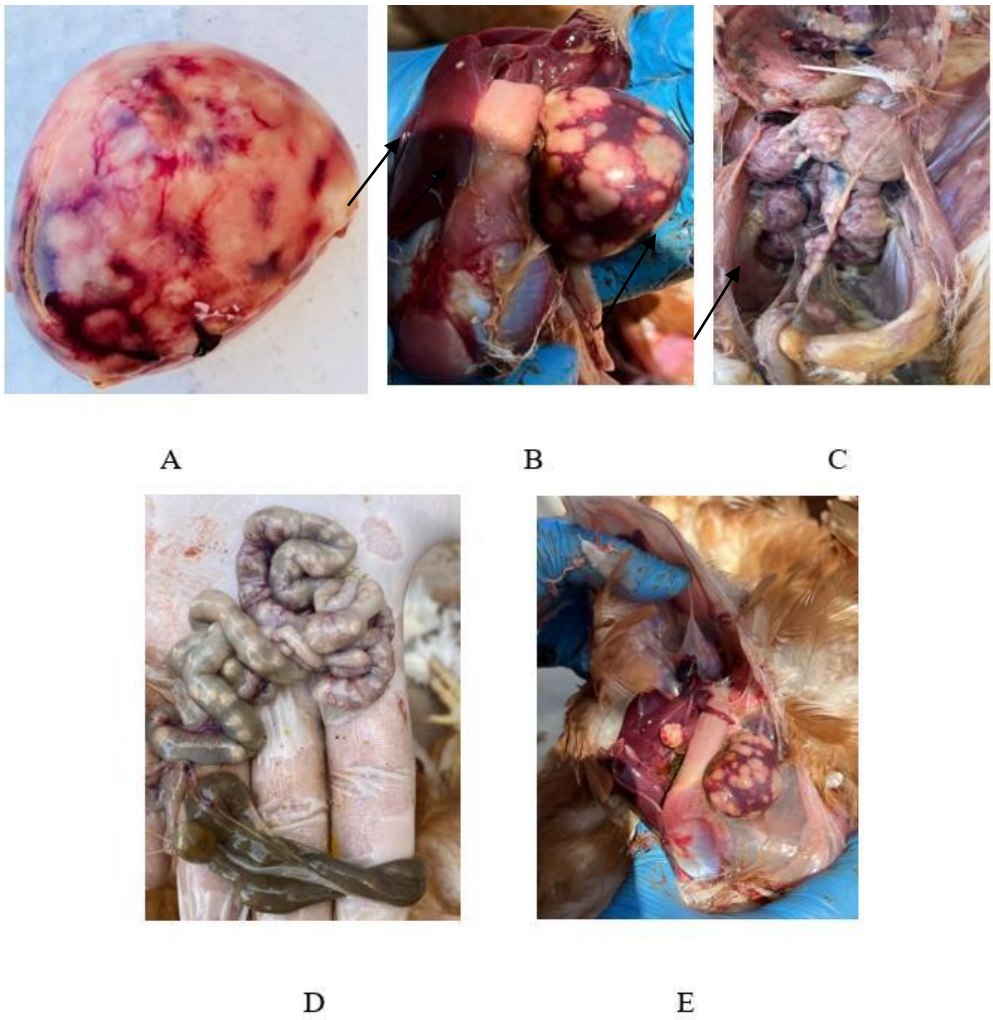


Figure 3. Visceral pathological lesion in organs of layer chickens with Marek's disease. A: Ovary in which there was severe enlargement with multiple whitish nodular lymphoid tumors which replaced the normal ovarian tissues (black arrows). B: Spleen with diagnostically important multifocal to coalescing whitish nodules (black arrows) suggestive of widespread lymphomatous infiltration. C: Proventriculus and adjacent viscera with large neoplastic nodules (black arrows) are seen along the serosal surfaces and mesolithic tissues. D: Intestines with congested and hemorrhagic serosal level (black arrows) with systemic involvement with severe vascular compromise because of Marek's disease. E: Liver and spleen with generalized tumor nodules (black arrows) having distorted organ architecture typical of the visceral form of Marek's diseas

As revealed by the ELISA, the two variants of NDV and IBV lowered the occurrence of the antibodies titers statistically (Tukey HSD, $p < 0.05$) in infected birds as compared to those in the control group. In particular, NDV antibody titer was the highest in the control group, and it was much higher than those in the infected White Lohmann and Brown Lohmann groups. The number of NDV titer titers showed no significant differences between the two infected groups. Similarly, IBV antibody titers were significantly higher in the control group compared to both infected White Lohmann and Brown Lohmann birds. Furthermore**,** no significant difference was observed between the two infected groups. (Table 2).

Tukey HSD results showed that there was significant difference ($p<0.001$) between the control and the two infected groups in terms of NDV and IBV antibody titers. No significant differences ($p>0.05$) were however found between White Lohmann and Brown Lohmann chickens.

Table 2. Comparison of NDV and IBV antibody titers (Mean ± SD) among groups (Tukey HSD, $p < 0.05$)

Group	NDV Antibody Titer (Mean ± SD)	IBV Antibody Titer (Mean ± SD)
Control Group	8650 ± 520 **a**	7800 ± 480 **a**

White Lohmann (infected)	5100 ± 430 **b**	4300 ± 390 **b**
Brown Lohmann (infected)	4700 ± 410 **b**	4000 ± 370 **b**

Note: Groups sharing the same letter are not significantly different; different letters indicate $p < 0.05$, and the HSD value 382 (Tukey HSD).

Histopathological showed that the incidence of lymphoid tumour infiltrations was significantly increased in all the tested organs (proventriculus, lung, heart, liver, and spleen) in both White Lohmann birds and Brown Lohmann birds than in the control group ($p < 0.001$, Tukey HSD). The lesions were again characteristic of the affected birds with 85 to 95 percent of the birds showing the same harboring the lesions as compared to all control birds that showed no lesions. Superscript letters denote that there is no significant difference ($p > 0.05$) between the infected groups in terms of the frequencies of lesions in all the organs, meaning that both the breeds have a similar histopathological reaction to MDV infection. Conversely, the comparison between the control and the infected groups in the assessed tissues was significantly different important directional ninety five percent of the comparison between the control and the infected groups in the assessed tissues was significantly different. These results have given solid statistical evidence to the pathological role of the Marek disease virus to cause the extensive lymphoproliferative lesions even irrespective of breed origins. (Table 3).

There were highly significant differences ($p < 0.05$) between the infected groups and control group in all gross lesions (Table 1) and histopathological lesions (Table 3). The HSD post-hoc test by Tukey established that the control group was significantly different to both infected groups, and there were no significant differences between White Lohmann and Brown Lohmann chickens, other than atrophic muscle where Brown Lohmann had a slightly higher prevalence . Likewise, the mean of NDV and IBV antibody titer were significantly lower ($p < 0.05$) in both the infected groups as compared to the control group (Table 2). Tables 1, 2 and 3 have included all the p-values and HSD to maintain statistical clarity and transparency.

Table 3. Histopathological lesions : frequency in affected and control groups

Organ	Histopathological Lesions (White Lohmann, n=100)	Histopathological Lesions (Brown Lohmann, n=100)	Control Group (n=100)
Proventriculus	95(95%)a	95 (95%)a	0 (0%)b
Lung	85 (85%)a	85 (90%)a	0 (0%)b
Heart	90 (90%)a	95 (95%)a	0 (0%)b
Liver	90 (90%)a	90 (90%)a	0 (0%)b
Spleen	95 (95%)a	95 (95%)a	0 (0%)b

Note: Different letters indicate significant differences between groups ($p < 0.05$); same letters mean no significant difference, and the HSD value = 1.89 (Tukey HSD).

Figure 4, 5, 6, 7 at 4x, 10x, 20x, 40x Magnification, the histopathological examination of the proventriculus under low and high-power microscopy revealed dense infiltration by lymphoid tumor cells interchanging normal glandular tissues. The sheets of sheets of neoplastic lymphocytes rendered the proventricular architecture inscrutable and destroyed the glandular parenchyma and normal epithelial elements. This lesion represents a visceral type of Marek's disease in which MDV-infected T-cells proliferate without control, invade epithelial and lymphoid organs. Being lymploid associated tissues rich, the proventriculus is a known target for lymphomagenesis induced by MDV. These conclusions showed that multifocal tumor development outside the usual nervous form had occurred due to the virus.

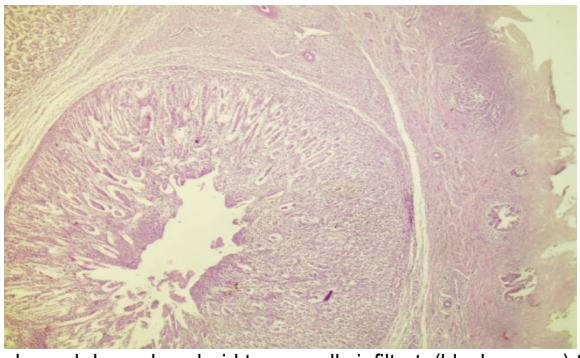


Figure 4. Proventriculus. This section showed dense lymphoid tumor cells infiltrate(black arrows) that effaced most of the proventriculus glands with loss of parenchyma through proventriculus glandular tissue. 4x. H&E.

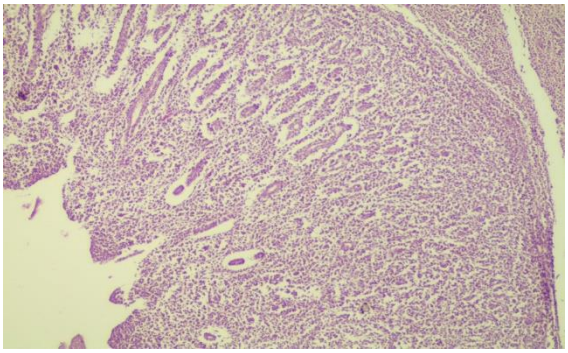


Figure 5. Proventriculus. This section showed dense lymphoid tumor cells infiltrate (black arrow) that effaced most of the proventriculus glands with loss of parenchyma through proventriculus glandular tissue. 10x. H&E.

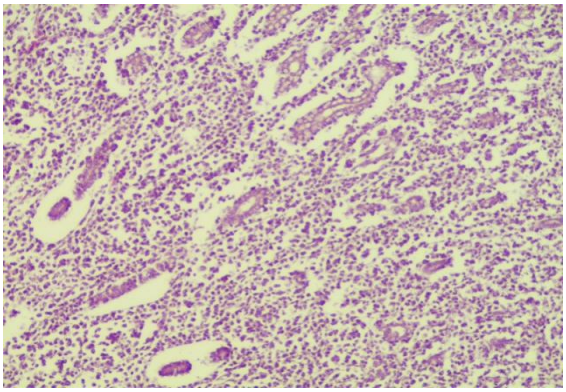


Figure 6. Proventriculus. This section showed dense lymphoid tumor cells infiltrate(black arrow) that effaced most of the proventriculus glands with loss of parenchyma through proventriculus glandular tissue.. 20x. H&E.

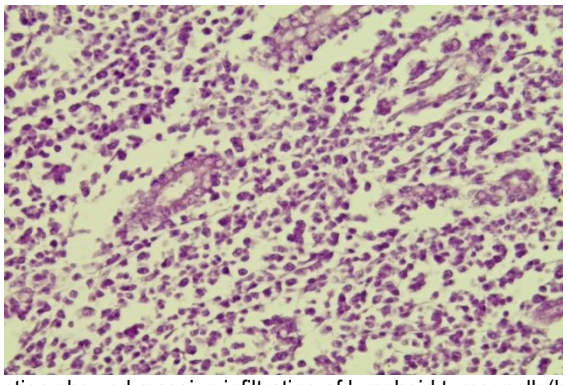


Figure7.Proventriculus. This section showed massive infiltration of lymphoid tumor cells(black arrow) in the necrotic glandular tissue with remnant of proventriculus glands. 40x. H&E

Figure 8, 9. 10, 11 at 4x, 10x, 20x, 40x magnification, in the lung sections mentioned, there were massive lymphoid tumor cell infiltration in the atria and parabronchial tissues with destruction of normal pulmonary architecture. The infiltrates squeezed and displaced air capillaries, crippling lung integrity to such a degree. These pulmonary lesions explain the clinical presentation of respiratory adversity as well as poor rates of oxygen exchange clinically. Marek's disease virus could spread hematogenously to the lungs, and lymphoid proliferation within pulmonary tissue will affect respiratory function. Like other visceral organs, the lungs function as secondary sites of tumor dissemination.

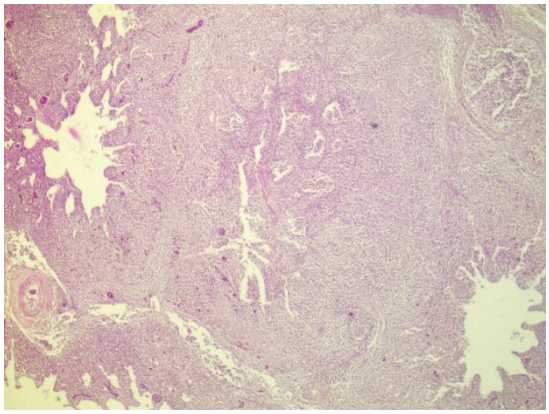


Figure 8. Lung. This section showed massive lymphoid tumor cells infiltrate(black arrow) the pulmonary parenchyma mainly in atria and parabronchial tissue with severe disruption of pulmonary tissue. 4x. H&E.

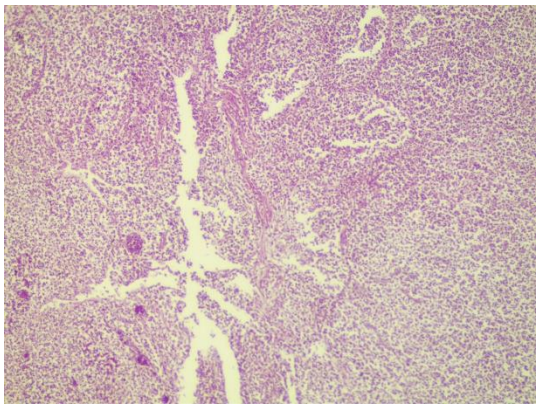


Figure 9. Lung. This section shows massive lymphoid tumor cells infiltrate(black arrow) the pulmonary parenchyma mainly in atria and parabronchial tissue with severe disruption of pulmonary tissue .10x. H&E.

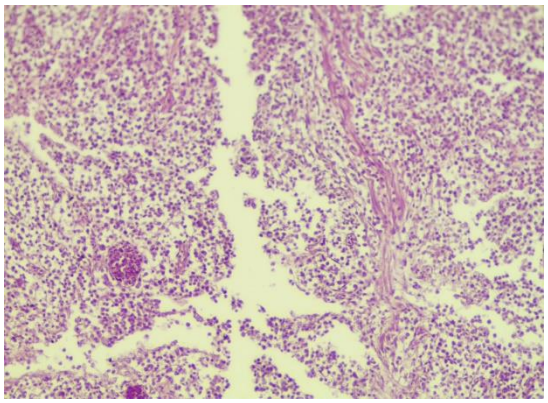


Figure 10. Lung. This section shows massive lymphoid tumor cells infiltrate(black arrow) the pulmonary parenchyma mainly in atria and parabronchial tissue with severe disruption of pulmonary tissue. 20x. H&E.

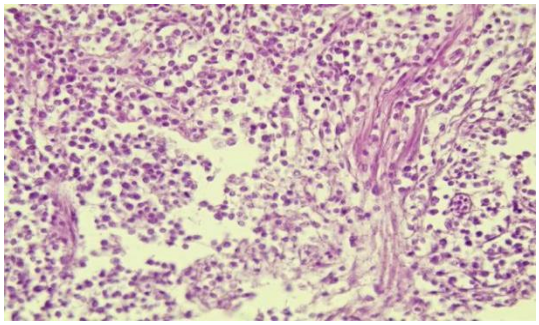


Figure 11. Lung. This section shows massive lymphoid tumor cells infiltrate(black arrow) the pulmonary parenchyma mainly in atria and parabronchial tissue with severe disruption of pulmonary tissu .40x. H&E.

Figure 12, 13, 14 at 10x, 20x, 40x magnification. Biopsies of the heart by microscopy showed infiltration of the lymphoid tumor cell between myocardial fibers with focal necrosis and myofiber degeneration. The neoplastic cells altered the striated pattern of cardiac muscle. The myocardial involvement is the infiltrative performance of MDV induced lymphomas into the mesenchymal tissues. It was this cardiac invasion that accounts for arrhythmias or heart failure signs sometimes observed in late stage cases of Marek's disease. Lymphoid proliferation replaces-cardiac tissue rendering it non-functional hence contractility is compromised.

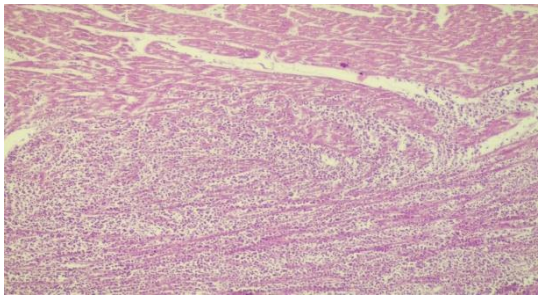


Figure 12. Heart. This section showed massive lymphoid tumor cells infiltrate(black arrow) myocardial fibers(red arrow) with evidence of severe muscle destruction. 10x. H&E.

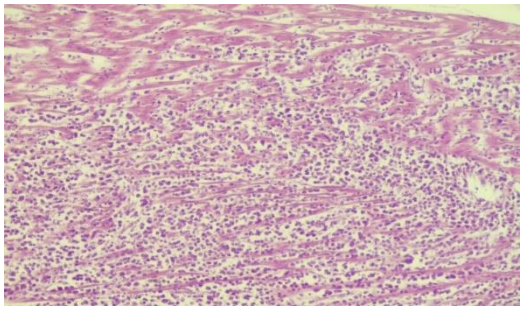


Figure 13. Heart. This section shows massive lymphoid tumor cells infiltrate(black arrow) myocardial fibers(red arrow) with evidence of severe muscle destruction. 20x. H&E.

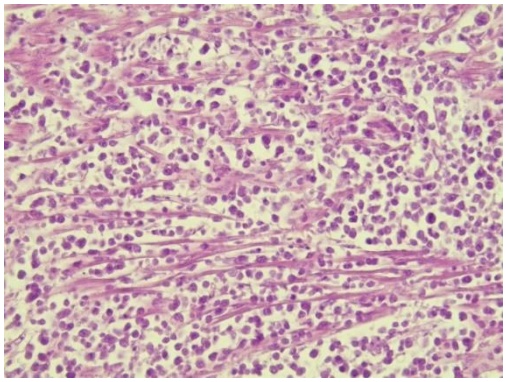


Figure 14. Heart. This section shows massive lymphoid tumor cells infiltrate(black arrow) myocardial fibers(red arrow) with evidence of severe muscle destruction. 40x. H&E.

Figure 15, 16 at 20x, 40x magnification, the liver sections showed diffuse infiltration of the lymphoid tumor cells that replaced the hepatic cords with atrophy of the hepatocytes and sinusoidal collapse. These hepatic changes were the signature of visceral Marek's disease and what it means for MDV to be oncogenic in the liver. Hepatic tumor infiltration might blister liver function and metabolism leading to emaciated bird with poor body condition.

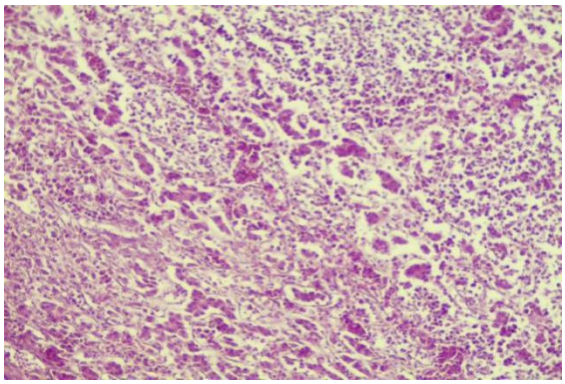


Figure 15. Liver. This section showed massive lymphoid tumor cells infiltrate(black arrow) liver parenchyma with marked atrophic hepatic cords(red arrow). 20x. H&E.

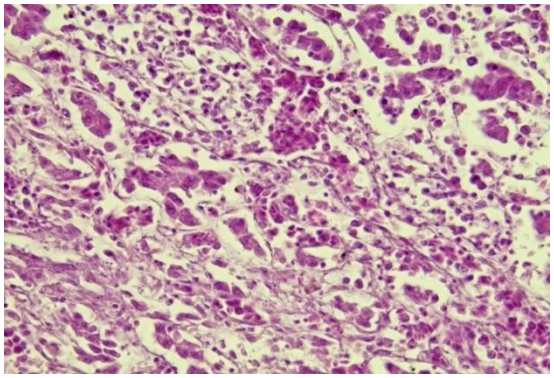


Figure 16. Liver. This section shows massive lymphoid tumor cells infiltrate(black arrow) liver parenchyma with marked atrophic hepatic cord(red arrow).. 40x. H&E.

Figure 17, 18, 19 at 10x, 20x, and 40x magnification, histological examination of the spleen revealed near complete replacement of normal splenic lymphoid tissue and red pulp of neoplastic lymphoid cells. The spleen was a major target for MDV-induced tumor formation, because it was an important lymphoid organ. Loss of normal lymphoid architecture was a surrogate marker of functional immunosuppression, reflective of the low antibody titers to NDV and IBV in serological assays. This spleen destruction interferes with bird's ability to mount protective immune responses.

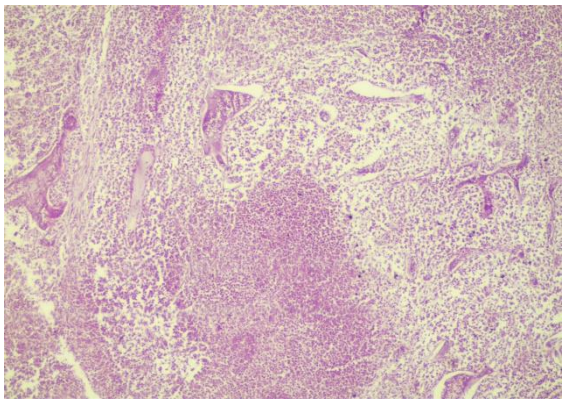


Figure 17. Spleen. This section shows massive lymphoid tumor cells infiltrate(black arrow) that replaced splenic lymphoid tissue and red pulp.10x. H&E.

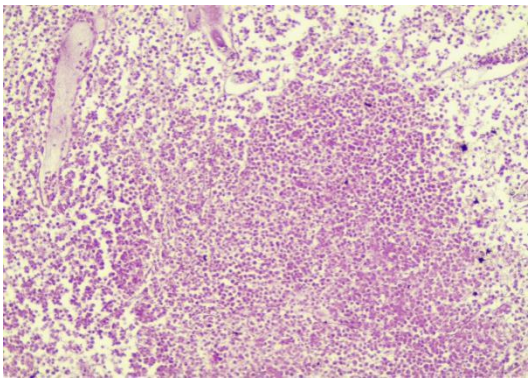


Figure 18. Spleen. This section shows massive lymphoid tumor cells infiltrate(black arrow) that replaced splenic lymphoid tissue and red pulp. 20x. H&E.

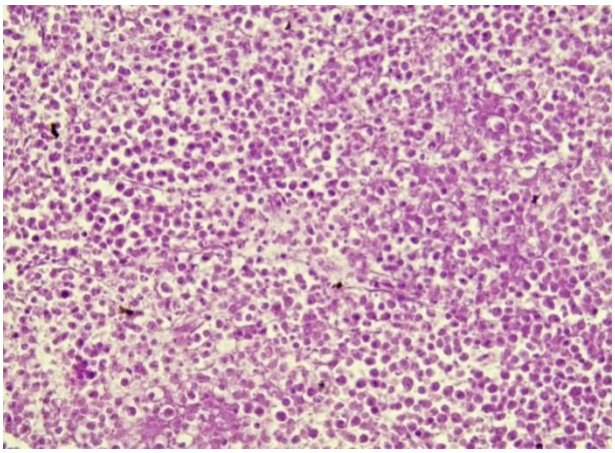


Figure 19. Spleen. This section showed massive lymphoid tumor cells infiltrate(black arrow) that replaced splenic lymphoid tissue. 40x. H&E.

PCR analysis targeting the Meq gene of Marek's disease virus (MDV) revealed an infection prevalence of 100% in both the White and Brown Lohmann groups, whereby out of 100 tested samples of each group, the corresponding PCR products were identified. Conversely, the amplification was not found in any of the samples of the control group. Agarose Gel electrophoresis successfully demonstrated amplicon size of 620base pairs with all positive samples portraying an apparent single amplicon at the expected size of 620 base pairs with regards to the *Meq* gene. The control group did not also show any bands, which also confirms the specificity of the assay and the healthy birds in the absence of MDV.**Table 4**.

Table 4. Detection of the *Meq* gene of Marek's disease virus (MDV) from tissue samples and feather swabs which were pooled together (meaning each sample represents the feather swabs, tissue samples of liver, sciatic nerve, and spleen of one chicken) by PCR and confirmation by agarose gel electrophoresis (620 bp).

Group	Number of Positive Samples from birds	Positivity Rate of carrying the Meq gene (%)	expected Band Size (bp)
White Lohmann	100	100%	620 bp
Brown Lohmann	100	100%	620 bp
Control Group	0	0%	-

4. Discussion

Central Finding

This study confirmed the presence of very virulent plus (vv+) MDV infection in vaccinated Brown and White Lohmann layer flocks in Basra, Iraq, demonstrating potential vaccine escape and breed-associated differences in susceptibility.

1. Clinical and Pathological Findings

The clinical signs, including asymmetric paralysis, wing drooping, progressive emaciation, and elevated mortality, correspond closely with classical manifestations of MD (14-16). Gross lesions, such as hepatosplenomegaly, kidney enlargment, and sciatic nerve swelling, were observed in over 85% of affected birds, with Brown Lohmann exhibiting slightly higher morality and incidence of nerve lesions than White Lohmann(17-19). These findings mirror observations from neighboring regions, including Turkey and Iran, where layer breeds displayed greater clinical severity and morality when infected with vv+ MDV strains (20-22).

2.

3. Histopathological Correlation

Microscopic analysis revealed widespread lymphoid tumor infiltration across multiple organs, including the proventriculus, lungs, heart, liver, and spleen, with near-complete replacement of splenic lymphoid tissue and red pulp(23). These lesions highlight the lymphotropic and neurotropic nature of MDV and correlate with the observed reduction in antibody titers against NDV and IBV, reflecting the immunosuppressive effect mediated by the Meq oncoprotein(24,25,). The slightly greater lymphoid infilitration in Brown Lohmann birds may reflect brees-specific genetic variation in immune response, particularly differences in major histocompatibility complex (MHC) alleles, which modulate T-cell mediated control of viral replication (26-28,).

4. Molecular Confirmation

PCR detection of the *Meg* gene in all infected birds confirmed the circulation of highly pathogenic vv+ MDV strains, consistent with reports of emerging variants capable of overcoming HVT-based vaccine protection (29-31,). These molecular findings reinforce the pathological observations, linking the presence of virulent MDV strains to tumor formation, lymphoid depletion and immunosuppression in the affected flocks.

5. Comparative Susceptibility Between Birds

Brown Lohmann birds exhibited slightly higher mortality and lower antibody titers than White Lohmann birds, suggesting greater field susceptibility. This may be attributed to breed-specific MHC variation affecting antiviral immune responses, as well as environmental factors such as stress, management practices, or nutrition, which can modulate immune competence. These results align with previous studies reporting breed-associated differences in MD outcomes and *Meq* gene expression (32-35,)

6. Field Implications and Control Strategy

The persistence of vv+ MDV in vaccinated flocks underscores the limitations of current HVT- based vaccines under field conditions. Continuous molecular surrveilience, rigorous evaluation of vaccine efficacy, and enhances biosecurity protocols are critical to mitigate MDV spread(36-38). Layer flocks, due to their longer lifespan and stronger immune responses, require targeted adaptive vaccination strategies to limit the impact of highly virulent strains and maintain production performance (39- 42)

5. Conclusion

This study demonstrates that Marek's disease virus (MDV) continues to represent a significant threat to commercial layer flocks in the Basra region, even among vaccinated populations. The integration of clinical, pathological, histopathological, serological, and molecular evidence provides compelling confirmation of the circulation of a highly virulent and immunosuppressive MDV strain capable of circumventing vaccine-induced protection. These findings emphasize the critical importance of ongoing molecular surrveilance, rigorous evaluation of vaccine performance, and the implementation of enhanced biosecurity protocols to effectively mitigate the re-emergence and transmission of MDV within poultry production systems. Brown Lohmann birds exhibited slightly higher mortality and lower antibody titers than White Lohmann birds, suggesting greater field susceptibility

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Conflicts of interest

There are no conflicts of interest.

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