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Insights into the Experimental Pathogenesis and Host Immune Response to a Backyard Poultry–Derived Marek's Disease Virus and the Efficacy of the CVI988 Rispens HVT Vaccine in Chickens

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MA and MRK designed and supervised the study. WA analyzed data, prepared visualizations and drafted the manuscript. HR and NYK assisted in experiments. SH and AZ performed laboratory work. SA and UR handled statistics and figures. TA and SUK reviewed the manuscript. HN provided technical guidance and approved the final version.

Key words
Marek's disease virus, Backyard poultry, CVI988/Rispens + HVT vaccine, Pathogenesis, Cytokine response, Viral load

ABSTRACT

Marek's disease virus (MDV) is a strongly tumor-inducing alphaherpesvirus and it continues to cause severe immunosuppression and mortality in poultry globally. This study aimed to evaluate the pathogenesis of a virulent MDV isolate from Punjab, Pakistan, in backyard poultry birds, and the protective efficacy of a commercial CVI988/Rispens strain-based HVT vaccine. Broiler chickens (n=160) were divided into four groups such as unvaccinated/infected (group A), vaccinated/infected (group B), vaccinated/uninfected (group C), and unvaccinated/uninfected controls (group D). Viral load was estimated in splenocytes by targeting the *meq* gene of MDV and the cytokines expression of IL-1β, IL-6, IL-8, IL-10, IL-18, IFN-γ, iNOS) was quantified by RT-qPCR. Histopathological scoring of major organs and statistical analysis (two-way ANOVA and Spearman's correlation) were performed. The results showed significantly higher viral loads in unvaccinated birds than in all other groups from 10 to 35 days post-infection (dpi) ($p < 0.05$). Cytokine expression varied significantly across the groups (e.g., IL-6, $p < 0.001$). Birds from unvaccinated group showed the highest early expression of iNOS (3 dpi, $p < 0.001$), IL-1β, and IFN-γ, indicating a pronounced innate response. Strong positive correlations were observed between spleen *meq* load and lesion scores in the liver ($r = 0.84$), kidney ($r = 0.76$), and heart ($r = 0.60$). Histological lesions were most severe in Group A, with median scores ranging from moderate (++) to severe (+++). The field MDV isolate caused extensive viral replication, cytokine dysregulation, and organ pathology. Vaccination with CVI988/Rispens + HVT effectively reduced viral load and immunopathology, affirming its protective efficacy under field-like conditions.

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INTRODUCTION

Marek's disease virus (MDV) belongs to the Herpesviridae family. It is a contagious viral disease primarily affecting chicken (Ozan *et al*., 2021). The global poultry industry faces substantial economic losses from Marek's disease (MD) because the disease causes higher mortality rates and deteriorates meat quality and reduces egg

production (Nawab *et al*., 2018). The management of this disease faces a major challenge because the virus continues to evolve which produces more dangerous strains (Nair, 2005). MDV exists in multiple pathotypes that include virulent strains which produce severe disease symptoms and strains that serve as bases for vaccine development (Shi *et al*., 2020). Understanding the pathogenesis of virulent MDV isolates is crucial for disease management and vaccine development. Effective control measures require identification and characterization of MDV strains because their virulence levels differ substantially (Deng *et al*., 2021).

The widespread use of vaccination as an MD prevention method has proven successful in reducing economic losses throughout the poultry industry (Mete *et al*., 2016). The Rispens strain vaccine administered to developing embryos through in-ovo delivery stands as the most effective approach. The vaccination process employs vaccines made from MDV-2 serotypes including SB-1 together with herpesvirus of turkeys (HVT) and the attenuated MDV-1 strain CVI988 (Rispens) (Gimeno, 2008). The HVT-based vaccine which is commonly used fails to provide adequate protection against MDV-1 variants that have become more virulent (Gimeno, 2008).

The infection process of MDV creates a complicated relationship between viral particles and immune systemof the host. The virus initially attacks T lymphocytes with a special focus on CD4+ T cells that serve as the main coordinators of immune responses. The infection of these cells leads to malignant transformation which produces lymphoma and severe immunosuppression (Yang *et al*., 2020). The weakened immune condition makes it difficult for the host to combat viral infections, resulting in an increased risk of developing secondary infections. According to Nedeva (2021) and Heidari *et al*. (2008) the activation of macrophages and cytotoxic T lymphocytes for antiviral defense depends on Th1 cytokines, including interferon-gamma (IFN-γ).

MDV infection leads to increased production of multiple cytokines in the body. The cytokines including IFN-α (Quere *et al*., 2005), IL-1β (Xing and Schat, 2000; Jarosinski *et al*., 2006), IL-6 (Abdul-Careem *et al*., 2007), IL-8 (Xing and Schat, 2000; Kaiser *et al*., 2003), IL-10 (Abdul-Careem *et al*., 2007), and IL-18 (Kaiser *et al*., 2003; Abdul-Careem *et al*., 2007) are elevated during MDV vaccination. The signature Th1 cytokine IFN-γ appears consistently in chickens infected with MDV according to previous research (Xing and Schat, 2000; Djeraba *et al*., 2002; Jarosinski *et al*., 2005). Notably, its expression is particularly elevated in the spleen during infection. Previous studies have shown that the expression of IFN-γ is downregulated in the peripheral blood cells of the susceptible lines, while it remains unaffected in resistant chicken lines (Quere *et al*., 2005; Xie *et al*., 2019). The present study aimed to investigate the experimental pathogenesis of a virulent Marek's disease virus (MDV) isolate obtained from backyard poultry under field conditions in Punjab, Pakistan, and to evaluate the effectiveness of commercially available vaccines. We also studied the relationship between viral load in their spleen tissues and the expression of immune response markers, including IL-1β, IL-6, IL-10, IFN-γ, IL-8, IL-18, and iNOS, at multiple time points post-infection.

MATERIALS AND METHODS

Vaccine and infection virus strains

The MDV virulent field strain (Accession No. MN923517.1) was present in our lab and used to infect chickens (Azeem *et al*., 2023). The vaccination administered was a bivalent one made up of CVI988/Rispens + HVT and provided by Merial, Inc., USA.

Experimental birds

One hundred and sixty fertile poultry eggs were kept in isolation and randomly assigned one of four groups on day 19th of incubation. The flock used in the study was confirmed to be free from major poultry diseases, and both feed and water were made available *ad-libitum*. The birds were randomly assigned to four experimental groups each of 40 birds. Group A received an intraperitoneal (i.p.) injection of 1000 PFU of a virulent field strain on the fifth day post-hatch. Group B was vaccinated on the first day after hatching with a combination of CVI988/Rispens and HVT via i.p. inoculation and subsequently infected by injecting 1000 PFU of the same virulent isolate 4 days post-vaccination. Group C was vaccinated using the same commercial formulation but was not challenged. Group D served as the unvaccinated, unchallenged control group. Birds were kept for 35 days after the challenge. Birds that died or were euthanized at the end of the trial were examined for tumor development. From the infected group, vaccinated-challenged group, vaccinated controls, and untreated controls, blood samples were collected in EDTA added vacutainers and splenic tissue samples were obtained from euthanized chicks from all the group at 3, 7, 10, 14, 21, and 35 dpi as previously described by Kano *et al*. (2009). On day 35 post-infection, 15 chicks were slaughtered, necropsied, and gross lesions indicative of MD were recorded. Density gradient centrifugation on Percoll® (Sigma-Aldrich) was used to separate PBMC and spleen cells. At each time point, three to five samples were harvested for each group.

Evaluation of viral load

Quantification of the MDV genome load in splenocytes was carried out using real-time PCR, utilizing DNA extracted from the samples (using Gene JET Genomic DNA Purification Kit Catalogue #KO721) and specific primers targeting the *meq* gene (184bp), as previously described by Abdul-Careem *et al*. (2006). The primer sequences were as follows: F = 5'-GTCCCCCCTCGATCTTTCTC-3' R= 5'-CGTCTGCTTCCTGCGTCTTC-3'. The amplification reactions were performed using the 7500 real-time PCR system (Applied Biosystems, Singapore). Each 20 µl reaction mixture consisted of PCR buffer, 2 µl of fast SYBR™ green master mix for PCR (Applied Biosystems, Singapore) and one microliter of each primer (0.2 µM). Thermal cycling involved initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles each of denaturation at 95°C for 10 sec, annealing at 64°C for 5 and an extension step at 72°C for 5 sec for *meq*).

Quantification of cytokines expression

TRI Reagent® was used to extract total RNA from isolated peripheral lymphocyte cells in accordance with the manufacturer's instructions. The harvested supernatants were kept at −80°C until further use (Kano *et al*., 2009). To ensure RNA purity, any residual genomic DNA was removed using DNase I (Thermo Scientific™). Subsequently, complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific™), following the manufacturer's protocol. Thermal cycling parameters were optimized based on the target gene. The protocol began with an initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles. These cycles typically included denaturation at 95°C for 10 sec (or 0 sec for IFN and IL-18), annealing at either 64°C for 5 sec or 55°C for genes such as IFN-γ, IL-6, β-actin, and IL-18, and an extension step at 72°C for 10 sec (with alternative durations of 5 sec for iNOS, 7 sec for IL-10). Melting curve analysis was conducted to verify amplification specificity, with steps including denaturation at 95°C for 0 sec, annealing at 65°C for 30 sec, and a final denaturation at 95°C for 0 sec, followed by a cooling phase at 40°C for 30 sec. Fluorescence signals were captured based on the optimal melting temperatures for each gene: IL-18, IFN-γ, and β-actin at 72°C for 10 sec; iNOS at 79°C for 10 sec; IL-10 at 80°C for 3 sec; IL-4 at 83°C for 3 sec; IL-12 p35 at 84°C for 3 sec; *meq* at 84°C for 10 sec; and IL-6 at 86°C for 3 sec. The complete list of specific primer sequences for the genes is provided in Table I.

Table I. Details of primers used in the study for real-time expression estimation of various cytokines.

Target genes	Primer Sequence	Product size	Reference
iNOS	F 5-GCACTACCTGCCTGGAGAAC-3	144bp	This study
	R 5-GCCCAATAGCCACCTTCAGT-3		
IL-1β	F 5- CTACAAGCTAAGTGGGCGCT-3	183bp	
	R 5- AAGCAACGGGACGGTAATGA -3		
IFN-γ	F 5-CTCCCGATGAACGACTTGAG-3	111bp	(Kano *et al.*, 2009)
	R 5-CTGAGACTGGCTCCTTTTCC-3		
IL-10	F 5-CATGCTGCTGGGCCTGAA-3	94bp	
	R 5-CGTCTCCTTGATCTGCTTGATG-3		
IL-6	F 5-CTGTTCGCCTTTCAGACCTACC-3	219bp	
	R 5-CATGGTGATTTTCTCTATCCAGTCC-3		
IL-8	F 5-CTGCGGTGCCAGTGCATTAG-3	139bp	
	R 5-AGCACACCTCTCTTCCATCC-3		
IL-18	F 5-GAAACGTCAATAGCCAGTTGC-3	213bp	
	R 5-TCCCATGCTCTTTCTCACAACA-3		
β-actin	F 5-CCAACTGGGATGATATGGAGAAG-3	204bp	
	R 5-AGGCATACAGGGACAGCACA-3		

Gross and histopathology

Lesions were evaluated based on the criteria outlined by Burgess *et al*. (2001). Lymphocytic infiltrations were assessed using 5 µm thick tissue sections of formalin-fixed, paraffin-embedded tissue from the heart, spleen, gonads, and liver stained with hematoxylin and eosin. For this study, a histological scoring system was applied: scattered lymphocytic infiltrations were assigned a score of (+), moderate multifocal lymphoid cell collections were given (++), and large, multifocal to coalescing sheets of lymphocytes that disrupted tissue architecture were rated (++), as per the method described by (Azeem *et al*., 2023). The histological scores for each section were compared to a predefined reference, and the cumulative scores were recorded accordingly.

Statistical analysis

Correlation analysis between spleen viral loads and histological lesion scores was analyzed using GraphPad Prism version 10.3 for Windows (GraphPad Software Inc., La Jolla, CA) (Ahmad *et al*., 2025). Differences among the various groups viral loads and cytokine expression profiles at different times were analyzed using two-way ANOVA. Statistical significance was considered as $P< 0.05$ (Ahmad *et al*., 2023).

RESULTS

MDV load in spleen cells

From the splenocytes of the chickens in each group at 3-, 7-, 10-, 14-, 21-, and 35-days post-infection, total cellular DNA was extracted. Real-time PCR was used to quantify the MDV genome loads (Fig. 1). All samples of Group A were identified with MDV genome. During the latent phase, *meq* gene concentration in Group A was momentarily elevated at 3 dpi and reduced at 7 dpi, respectively.

Group A also showed significantly higher gene loads than the other groups at 10 dpi, 14 dpi, 21 dpi, and 35 dpi. Group A had a significantly higher gene load than all other groups ($p < 0.05$). In contrast, Groups B, C, and D consistently displayed no detectable *meq* gene load throughout the study period, and these groups did not differ significantly.

Cytokine profiles of vaccinated and virulent MDV-infected chickens

Our results revealed significant differences in iNOS expression between the groups and over time. At 3dpi, Group A exhibited the highest iNOS expression, significantly different from all other groups. By 7dpi, iNOS expression in Group A decreased significantly but

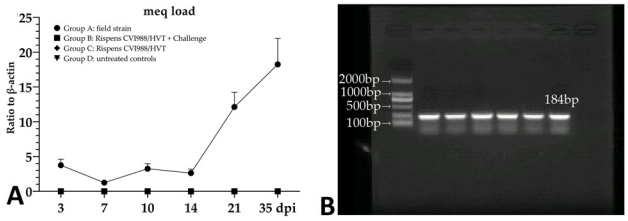

Fig. 1. (A) Quantification of *meq* gene in spleen cells of infected chickens using real-time PCR analysis. The outcomes are displayed as ratios between the chicken β-actin and *meq* gene concentrations. (B) Gel electrophoresis of the real-time PCR showing 184bp product.

remained higher than in Group B, Group C, and Group D. At 10dpi, Group A and Group B showed higher iNOS expression than Group C and Group D. However, from 14dpi onwards, there were no significant differences in iNOS expression between the groups (Fig. 2A). At 3dpi and 7dpi, Group A exhibited significantly elevated IL-1β levels compared to Groups B, C, and D. At 10dpi, Group B had the highest IL-1β levels, significantly differing from all other groups. At 14dpi, Group C had significantly greater IL-1β levels compared to Group D. At 21dpi, Group C had significantly elevated IL-1β levels than Groups A and D. Lastly, at 35dpi, Group C had significantly higher IL-1β levels than Groups A and D (Fig. 2B). Our analysis revealed significant differences in IL-6 expression among the various treatment groups and time points (3, 7, 10, 14, 21, and 35 days post-infection). The data is represented as mean ± standard error, and different alphabetical superscripts denote significant differences between individual groups. Group A exhibited a substantial increase in IL-6 expression at 3 days post-infection (dpi) compared to all other time points (a), while Group B showed a significant increase at 7 dpi (b). Group C remarkably increased IL-6 levels at 14 dpi (a), and Group D displayed a significant increase at 10 dpi (d). These results indicate that IL-6 expression varied significantly across different groups and time points, highlighting the dynamic nature of the immune response in PBMC under these experimental conditions (Fig. 2C). The results regarding IL-10 showed significant differences in IL-10 levels among the treatment groups ($p < 0.001$). Group B also showed significant differences from Group A and Group D at several time points. However, Group A and Group D were not significantly different (Fig. 2D). Group A, IFN-γ levels at 3dpi and 14dpi were significantly different from those at 10dpi and 21dpi, suggesting variations in the immune response over time within this group. Similarly, similar significant differences were observed in other groups, highlighting the dynamic changes in IFN-γ levels in response to different groups (Fig. 2E). Results regarding

IL-8 expression levels showed that at 3dpi, Group B has the highest IL-8 levels (7.4±0.46), significantly different from all other groups ($p < 0.05$), while Group A (2.5±0.27) is significantly different from Group C and Group D ($p < 0.05$). At 7dpi, Group B (10±0.84) remains significantly higher than the others ($p < 0.05$). Groups A, C, and D showed no significant differences. Similar patterns of significance are observed at 10dpi, 14dpi, and 21dpi, with Group B consistently having higher IL-8 levels. At 35dpi, Group B and Group C have the highest levels, significantly different from Group A and D (Fig. 2F). At 3dpi, Group A showed significantly higher IL-18 levels (5.2±0.37) compared to Groups B, C, and D (0.5±0.19, 4.6±0.37, and 0±0, respectively), denoted by different letters. Similarly, at 7dpi, Group A (0.5±0.19) significantly differed from Groups B and C (3.6±0.32 and 4.4±0.26, respectively). However, by 10dpi, IL-18 levels in Group A (0.38±0.18) were significantly lower than in Groups B, C, and D (3.2±0.45, 3.1±0.48, and 0, respectively). At 14dpi, 21dpi, and 35dpi, Group B consistently showed the highest IL-18 levels, significantly different from other groups. Group C also showed significant differences from Group A at 14dpi and 21dpi (Fig. 2G).

Several intriguing relationships have emerged in the correlation analysis of the *meq* gene load with various cytokines. Notably, a direct correlation was observed between meq gene load and IFN-γ ($R = 0.3077$), indicating a moderate positive association between meq load and IFN-γ levels. Conversely, there were negative correlations between *meq* gene load and several cytokines, including iNOS ($R = -0.2359$), IL-18 ($R = -0.2360$), IL-1β ($R = -0.0896$), IL-6 ($R = -0.0229$), IL-8 ($R = -0.1433$), and IL-10 ($R = -0.0182$), suggesting an inverse relationship wherein increased *meq* gene load was associated with decreased levels of these cytokines. These findings suggest a potential immunosuppressive effect associated with a higher *meq* gene load (Fig. 3).

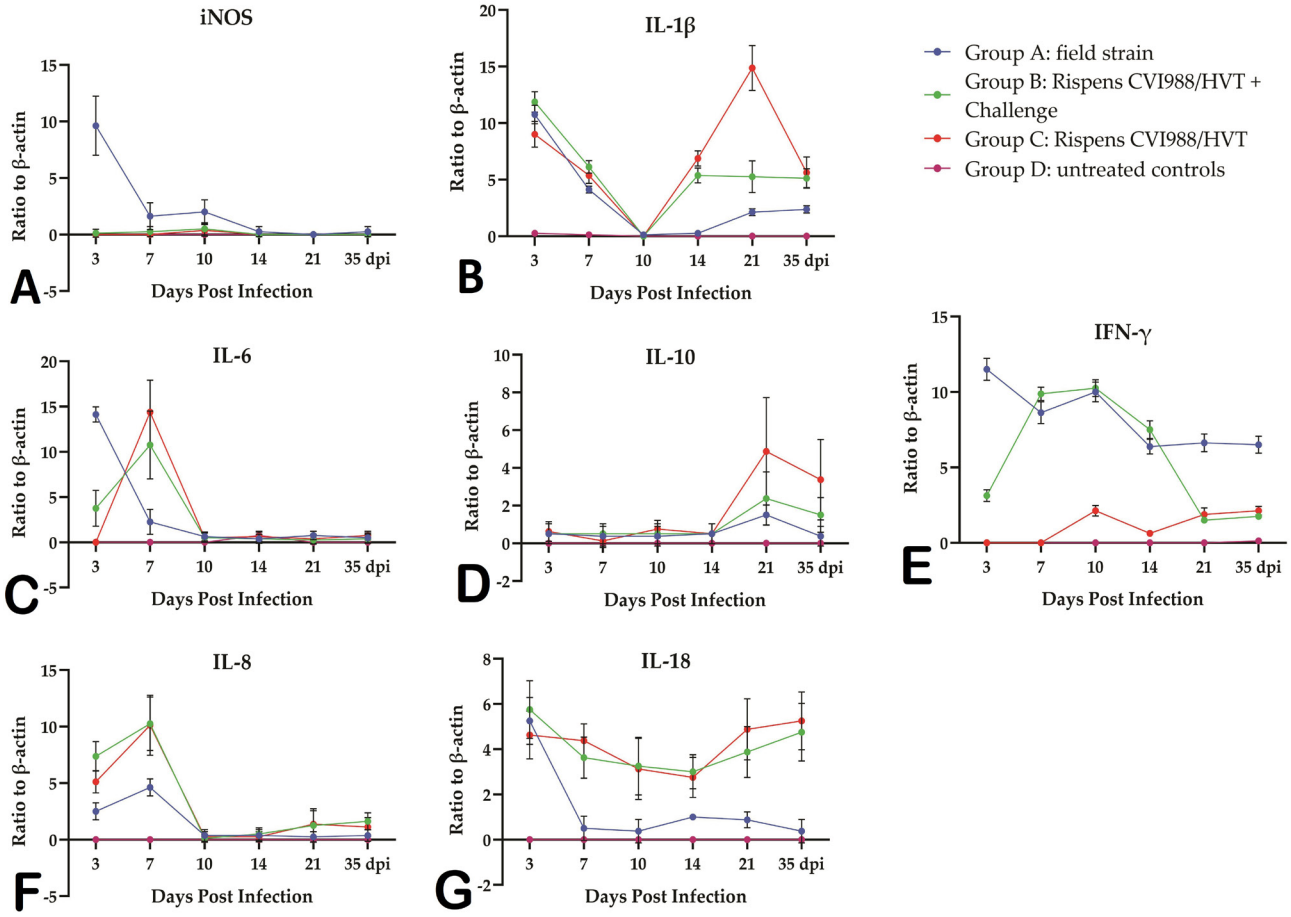

Fig. 2. The cytokines mRNA expression in splenocytes from experimental chicken groups. An amount of gene expression is expressed in relation to β-actin.

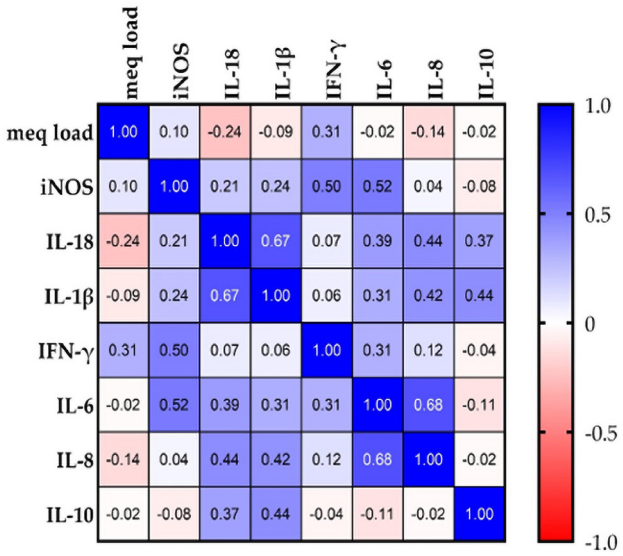

Fig. 3. Correlation matrix of the relationships between the *meq* gene load and various cytokines, including iNOS, IL-6, IL-18, IFN-γ, IL-1β, IL-8, and IL-10. The values in the cells represent the correlation coefficients (R-values) that quantitatively reflect the strength and direction of the associations between these variables. The positive and negative correlations indicate the degree to which changes in *meq* gene load are associated with cytokine levels.

Prior to 21 days postpartum, no chicken in either group had any clinical signs. Nonetheless, Group A chickens exhibited every MD characteristic on the 23–35 dpi Groups A and B chickens showed splenomegaly from 3 dpi, with Group A having the highest degree of splenomegaly compared to the other groups. A histological analysis revealed lymphoproliferation in the kidney, spleen, liver, heart, and gonads (Fig. 4).

There was a range in these lymphoproliferative alterations from mild (+) to moderate (++) to severe (+++). In a few splenic tissues, lymphoid tissue completely replaced the parenchyma. The tumorous lymphocytes ranged in size, with a noticeable consistency in their sizes. These cells replaced sections of the liver and spleen parenchyma, varying in size from multifocal to scattered. Plasmodic lymphoblastic and lymphocytic cells, which make up most of the organ's cells, caused the organ's usual consistency to be upset. The spleen tissues have moderate (++) median histopathological scores. In addition, the severe (+++) histopathology grades of 95% of the subjects were false. Both localized and widespread pleomorphic cell infiltration were visible in the liver. Mixtures of plasma cells, macrophages, small-to-medium-sized lymphocytes, and lymphoblasts comprise these cells. There were also a few plasma cells and a few macrophages. The number of mitotic figures of the infiltrating lymphoblasts was considerable. Lymphocytes infiltrate the cell and replace the natural parenchyma. Additionally, perivascular lymphoblast infiltration was noted. The liver tissue's median histology score was moderate (++). Conversely, the hepatic parenchyma of 95 percentile individuals showed substantial (+++) alterations. The median mononuclear cells infiltration score in cardiac tissues was slight (+), with the 95% and 5% percentiles of individuals ranging between the moderate (++) and normal ranges. The 95 percentile people had normal and severe (+++) histology scores, respectively, while the gonads showed the median of mild (+) histological alterations (Table II).

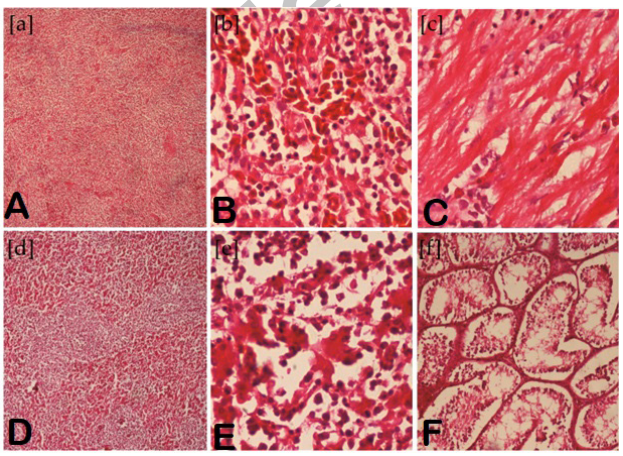

Fig. 4. Histological structure of spleen (A, B), myocytes (C), hepatic tissue (D, E) and testis (F) damaged affected showing areas. Stain: H & E. Panels A and D were seen at 10×, while B, C, E and F were observed at 100×.

The Spearman ranked correlation analysis reveals the relationship between the severity of lymphocytic proliferation in various organs, as indicated by the *meq* load (Table III). Notably, there is a strong positive correlation between *meq* load in the spleen and *meq* load in the liver (r= 0.84) and a moderately positive correlation between *meq* load in the spleen and *meq* load in the kidney (r= 0.76). These findings suggest a close association between the severity of lymphocytic proliferation in these organ systems, potentially indicating a shared pathological mechanism or similar susceptibility to lymphocytic proliferation. On the other hand, there is a relatively weaker correlation between *meq* load in the spleen and *meq* load in the heart (r= 0.60), and between *meq* load in the spleen and *meq* load in the gonads (r= 0.70) (Table III).

Table II. Histopathological scores of the 15 birds of Group A infected with MDV, which were slaughtered at the 35 d.p.i. The lesion scoring is labelled as healthy/ normal tissue (-), mildly affected (+), moderately affected (++), and severely affected (+++).

Viral load	Histological scores				
	Spleen	Heart	Kidney	Liver	Gonads
13	++	++	+++	+++	+
13	++	+++	+	++	+++
14	+++	+++	+	+++	+
14	++	+	+++	+	++
14	+++	+++	+	++	+
15	+++	+	+++	++	+
15	+++	++	+++	++	+
16	+++	+++	+++	+++	++
16	+++	+	+	++	+++
17	+++	+++	+++	+++	++
17	++	+++	+++	++	++
18	+++	++	+	+++	+
18	+++	+	++	+++	+++
19	+++	++	++	++	+++
20	+++	++	+++	+++	+++

Table III. Matrix that shows the relationship between the infiltration scores of lymphocytes in the body tissues of infected birds and the *meq* gene concentration. R was determined for each variable using Spearman's ranked correlation.

	***meq* load**	**Go-nads**	**Heart**	**Spleen**	**Liver**	**Kidney**	**P value**
meq load	-	0.70	0.60	0.84	0.81	0.76	<0.001
Heart	0.60	0.42	-	0.54	0.67	0.41	
Spleen	0.84	0.51	0.54	-	0.73	0.62	
Liver	0.81	0.51	0.67	0.73	-	0.60	
Kidney	0.76	0.43	0.41	0.62	0.60	-	
Gonads	0.70	-	0.42	0.51	0.51	0.43	

DISCUSSION

Over the past several decades, MDV has evolved to become increasingly virulent, prompting researchers across the globe to develop new vaccines based on contemporary viral strains. Despite extensive efforts, the precise mechanisms driving this progressive rise in virulence remain unclear, although numerous interconnected factors are believed to contribute (Jarosinski *et al*., 2006). One contributing factor might be the administration of suboptimal vaccine doses, which can lead to reduced immune responses compared to those achieved with the recommended vaccine quantity (Zimmermann and Curtis, 2019).

One of the key observations from this study is the differential viral load in chickens infected with MDV. Group A consisted of the unvaccinated birds which were experimentally exposed to virulent MDV and exhibited a distinct viral load pattern as compared to the other groups (B, C, and D). Notably, a momentary increase in MDV genome load was recorded in this group at 3 dpi, followed by a decrease at seven dpi during the latent phase. Subsequently, Group A maintained significantly higher MDV gene loads compared to other groups at dpi 10, 14, 21, and 35. This is suggestive of a higher viral replication rate and propagation in Group A chickens throughout the study period. In contrast, Groups B, C, and D consistently displayed no detectable *meq* gene load during the study.

The real-time polymerase chain reaction is an effective method for assessing the intensity of MDV infection and the likelihood of MD induced proliferation of lymphocytes development by quantifying the *meq* gene concentration in the infected tissues (Abdul-Careem *et al*., 2006). The cytokine profiles examined in this study are suggestive of the host immune response to MDV infection. The dynamics of cytokine expression particularly IFN-γ, IL-6, IL-8, IL-1β, IL-10, and IL-18, revealed distinct patterns during the course of infection. Group A chickens exhibited significantly higher expression of iNOS, IFN-γ and IL-1β at the early stages of infection (3 dpi and 7 dpi), indicating a robust initial immune response. Notably, iNOS expression in Group A was although it decreased after 7 dpi but remained higher than the other groups. IL-6 expression varied significantly among groups and various time points. Moreover, each of the groups exhibited unique patterns of IL-6 expression at each time points. A noticeable increase in IFN-γ that is a key Th1-type cytokine was observed in peripheral blood mononuclear cells (PBMCs) of vaccinated chickens after exposure to a virulent strain of MDV. The function of chicken IFN-γ is largely consistent with that of its mammalian counterparts, as outlined in earlier studies (Digby and Lowenthal, 1995; Lowenthal *et al*., 1995; Weining *et al*., 1996). This cytokine plays an essential role to control MDV replication, largely through the activation of macrophages, which then produce nitric oxide (NO) a molecule known for its ability to damage virus-infected cells (Lee *et al*., 1978; Djeraba *et al*., 2000; Jarosinski *et al*., 2005). Moreover, IFN-γ is functionally associated with natural killer (NK) cells and CD8+ T cells, both of which play role in elimination of MDV-infected cells through cytotoxic activity. Vaccination with HVT, a combination

of HVT and SB-1, or CVI988 has been shown to increase the population of NK and CD8+ T cell (May *et al*., 2000; Tahir *et al*., 2025).

The timing of induction of IFN-γ appears to be a critical factor in controlling MDV, especially in curbing the reactivation of latent virus, as demonstrated in murine gamma herpesvirus models (Steed *et al*., 2007). In unvaccinated chickens challenged with MDV. However, mRNA levels of IFN-γ during the initial latent phase did not correlate with viral genome loads. This persistent expression could be attributed to transient activation of CD4+ cells, which are main targets of MDV latency and transformation (Laursen, 2017). On the other hand, vaccination seems to promote a more effective IFN-γ response during latency, potentially enhancing protection against disease progression.

Interestingly, even though mRNA of IFN-γ was detectable in Group A birds through latency period, but viral reactivation could not be prevented. This might be due to reduced expression of IFN-γ receptor subunit 2 (IFNGR2) and interferon regulatory factor 3 (IRF-3), whose functions in chickens remain incompletely understood (Kano *et al*., 2009). NO, which is produced predominantly by macrophages the initial targets of MDV infection has demonstrated an ability to suppress MDV replication *in vivo* (Mehrzad *et al*., 2024). However, the macrophage-mediated immune response may play a less significant role during the latent phase. IFN-γ also modulates various immune cell subsets including NK cells, CD8+ αβ and γδ T cells, and macrophages, all of which are thought to contribute to host defense, particularly in the later stages of MDV infection (Katneni, 2015). Through the cytolytic phase, virulent MDV induces a marked suppression in the expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and IL-18, which is indicative of MDV-induced immunosuppression (Neerukonda, 2018). NO produced via inducible nitric oxide synthase (iNOS) may contribute to this immunosuppression by limiting T-cell proliferation and inflicting collateral damage on host tissues (van der Veen, 2001). Although IL-18 is known to enhance IFN-γ production, it may also drive Th2-associated cytokine responses in murine models (Osaki *et al*., 1999; Yang Jianfei *et al*., 2001), suggesting a complex role in immune regulation during MDV infection.

The lesions observed in this study closely align with those previously reported (Hayajenh *et al*., 2021; Zelník, 2021; Azeem *et al*., 2023). Similarly, Vieira-Pinto *et al*. (2003) emphasized the utility of histopathological evaluation in confirming the diagnosis of MD. In cases of the subclinical form, histological examination of liver and spleen tissues revealed neoplastic foci accompanied by pleomorphic cells. Additionally, lymphomatous infiltrates were evident in certain tissue regions, predominantly composed of lymphocytes and lymphoblasts. These infiltrative lesions typically appeared elongated, varying from small to medium in size. While the tumor microenvironment remained largely consistent across different organs, the severity and extent of involvement differed (Stamilla *et al*., 2020; Khan *et al*., 2021). Researchers further detected MD-specific features within the nuclei of neoplastic lymphoid cells in hepatic tissues (Wen *et al*., 2018). The occurrences of MD in vaccinated poultry populations were also documented by (Zelnik *et al*., 2004). Khan *et al*. (2021) noted that in infected livers, infiltrative cell clusters frequently surrounded smaller blood vessels, with tumor architecture dominated by proliferating malignant cells. Histopathological analysis of the visceral organs, including the spleen, liver, heart, and gonads, revealed a range of lymphoproliferative abnormalities. Notably, Group A chickens displayed more severe splenomegaly compared to the other groups, consistent with the higher viral load observed in this group. The histological scores indicated substantial lymphocytic infiltration in various organs, with severity ranging from mild to severe. Furthermore, a strong positive correlation was observed between *meq* gene load in the spleen and *meq* gene load in the liver, suggesting a close association between the severity of lymphocytic proliferation in these organs. This correlation potentially indicates a shared pathological mechanism or similar susceptibility to lymphocytic proliferation in the spleen and liver (Stamilla *et al*., 2020; Khan *et al*., 2021).

Future studies should explore the molecular mechanisms underlying the immune modulation caused by virulent MDV field strains. A long-term field trial to evaluate vaccine efficacy across different poultry breeds and environmental conditions is also recommended to validate the generalizability of these findings.

CONCLUSIONS

It was concluded that chicken infected with the virulent MDV strain prevalent under field conditions of Pakistan results in significant viral multiplication, dysregulation of immune responses in splenocytes and marked histopathological alterations in multiple organs. Vaccination with the CVI988/Rispens based HVT vaccine effectively mitigated the viral loads and immune dysregulation, further suggesting the vaccine's efficacy in controlling MDV infection under field conditions in Punjab, Pakistan.

DECLARATIONS

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IRB approval

The present study was conducted after approval by the Institutional Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan (DR/209, 24th April, 2019) in accordance with Declaration of Helsinki for animals.

Generative AI and AI-assisted technology statement

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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