

## Marek's disease in backyard chickens of Pakistan: A study of pathological lesions in correlation to viral load

M. Azeem<sup>1</sup>, M. ur Rehman Khan<sup>1#</sup>, G. Saleem<sup>1</sup>, M. Hassan Mushtaq<sup>2</sup>

<sup>1</sup> Department of Pathology, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan

<sup>2</sup> Department of Epidemiology & Public Health, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan

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### Abstract

Marek's disease (MD) is an infectious, lymphoproliferative disorder caused by the alpha herpesvirus genus, *Mardivirus*, serotype 1 (Gallid Herpesvirus 2, or GaHV-2). There is no current information on Marek's disease virus (MDV) pathotypes and serotypes circulating in backyard poultry in Pakistan. This study aimed to characterize GaHV-2 in the backyard poultry of Rawalpindi division based on glycoprotein C and to investigate the correlation of a viral oncogene (*meq*) with the histological, lymphoproliferative infiltration in the visceral organs of naturally-infected birds. The study was performed from May 2019 to April 2020 in the Rawalpindi, Jhelum, Chakwal, and Attock districts of the Rawalpindi division. An overall 13.96% prevalence of MDV infection based on gross morphology and 18.43% using polymerase chain reaction (PCR) was recorded. The Pakistani GaHV-2 isolates could be divided into two clades, based on sequencing and phylogenetic analysis of the *gC* gene, closely related to the US virulent isolates and mildly virulent vaccine strain, CVI988, respectively. Of total PCR-positive cases, 25 (75.76%) samples revealed gross tumours. Median histopathological scores of lymphoproliferative infiltrations in the spleen and liver tissues were recorded as moderate (++) , whereas in the heart and gonads, mild (+) changes were observed. The mean viral load quantified using real-time PCR of 14.72 copies of *meq* gene/ $\beta$ -*actin* revealed a strong ( $R^2 = 0.88$ ) correlation with histopathology scores of spleen tissues. Differences in the *meq* gene/ $\beta$ -*actin* ratio were observed between non-tumorous and tumorous birds, which is indicative of pathological changes in individuals of the present study.

**Keywords:** alpha herpesvirus, glycoprotein C, histopathology scores, *meq*-oncogene, Rawalpindi division, real-time polymerase chain reaction, Gallid herpesvirus-2

#Corresponding author: [muhammad.azeem@uvas.edu.pk](mailto:muhammad.azeem@uvas.edu.pk)

### Introduction

Marek's disease (MD) is caused by Marek's disease virus (MDV), which consists of three serotypes; MDV-1 is the pathogenic form, whereas MDV-2 and MDV-3 (herpesvirus of turkeys; HVT) are non-pathogenic and primarily used as protective immunizations against MDV-1. MD is a cell-associated, alpha herpesvirus demonstrated as the fifth most common infectious disease in chickens in a backyard poultry disorder survey (Mete *et al.*, 2016). The oncogenic MDV-1 virus can cause infection in 1-day-old chicks. The first phase of this process is the cytolitic phase, followed by latency. The second cytolitic phase occurs next, transforming the T lymphocytes of the affected host, leading to the development of lymphoproliferative disease or lymphomas (Payne & Nair, 2012).

Vaccination is the primary method of preventing MD, and strategies such as the widespread usage of vaccines within the poultry industry have been relatively successful in lowering economic losses (Mete *et al.*, 2016). The most effective way to administer vaccines created from serotypes MDV-

2 (such as SB-1), HVT, and attenuated MDV-1 (such as CVI988; Rispens) is through *in-ovo* vaccination with Rispens (Gimeno, 2008). Vaccine failures can be caused by prior virus exposure, inadequate handling, and administration of the vaccine, or weakened immunity (due to insufficient maternal antibodies or concurrent infections) in the chicken (Baigent *et al.*, 2006; Dunn *et al.*, 2014). Furthermore, the emergence of more virulent strains (virulent pathotypes) of MDV-1 have been linked to the failure of some vaccines (Witter, 1997; Nair, 2005), such that the most commonly-used HVT vaccine does not provide adequate immunization against very virulent MDV-1 (Gimeno, 2008).

Surveys and on-field experience indicate that backyard chicken farmers do not understand vaccination protocols for MD (Lister & Houghton-Wallace, 2012; Elkhoraibi *et al.*, 2014; Whitehead & Roberts, 2014). MDV-1 pathotype has been studied in chickens, primarily in commercial poultry lines under laboratory conditions. Such settings often use genetically susceptible or resistant chickens that lack antibodies and are referred to as specific-pathogen-free or antibody-free chickens to observe the progress of the disease, its viral pathogenesis, and the efficacy of vaccines (Bacon *et al.*, 2001a).

Diagnosing MD in backyard chickens is complex due to the wide variety of host lineages and infective MDV pathotypes. Additionally, the clinical signs and lesions of MD may be similar to those of other lymphoproliferative diseases or even peripheral neuropathy, an autoimmune disorder (Bacon *et al.*, 2001b; Witter *et al.*, 2010). Researchers have identified several criteria that can help distinguish MD in chickens, including the age of the chicken, changes observed under a microscope, immunohistochemistry tests, and molecular techniques. Nevertheless, multiple combined methods are often necessary to properly diagnose the condition (Witter *et al.*, 2010). Glycoprotein C (gC) is a valuable diagnostic tool in MD, an essential avian virus known to cause severe neurological disease and death in poultry. Further, analysis of the glycoprotein profile can be used to differentiate between the genotypes of each MD virus strain, ultimately leading to improved targeted strategies to combat this essential avian pathogen (Jarosinski & Osterrieder, 2012; Hassanin *et al.*, 2013).

Real-time quantitative polymerase chain reaction (qPCR) analysis is suggested as a definitive diagnostic tool for tumours where the MDV load is substantially higher than in nontumorous tissues. As a result, diagnosing MD in birds of varying or unknown ages and vaccination histories with an extensive overlap of lesions poses a unique challenge for diagnosticians in the field, who lack the necessary resources. This need is especially notable in settings where individual poultry health monitoring is prioritized over the flock approach.

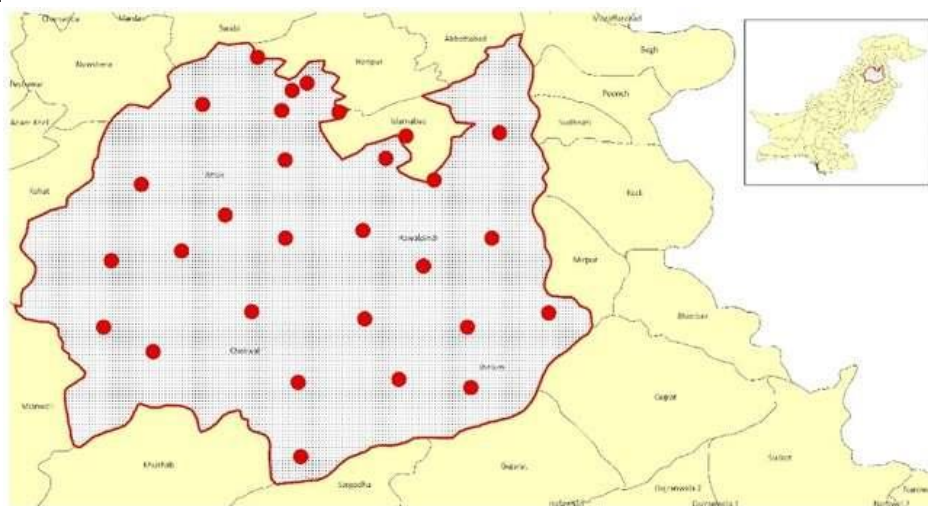
The present study aimed to study the phylogenetic analysis of MDV tumorous and non-tumorous backyard chickens of Rawalpindi division, and to correlate the histopathologic scores and MDV-1 viral loads.

## Materials and Methods

The study was conducted after approval from the research ethical guidelines committee, University of Veterinary and Animal Sciences, Lahore (DR/209, 24<sup>th</sup> April, 2019).

The samples were collected from May 2019 to April 2020 from 40 backyard chicken flocks (179 birds) from four districts in Rawalpindi Division: Rawalpindi, Jhelum, Chakwal, and Attock districts (Figure 1). The samples were taken from two to three birds per flock. Samples were taken from each bird's visceral organs with diffuse or localized tumours. The samples were kept at -20 °C until the DNA was extracted. Information regarding age, sex, breed, clinical signs, and vaccination status were also collected. Based on the owners' information, the vaccinated birds had a history of inoculation on day 1

at the hatchery level with commercially-available, live cell-associated vaccine (Rispens+HVT, Merial, Inc., USA).



**Figure 1** Map of Pakistan with Rawalpindi division comprising of the four districts, developed using ArcMap 10.3 software. Red dots show the localization of the sampling sites in the district

Total cellular DNA was extracted using the ThermoScientific® GeneJET Genomic DNA Purification kit, according to the manufacturer's instructions. PCR was conducted to detect a 686-bp glycoprotein C (gC) amplicon from Gallid alpha herpes virus type 1, with primers and conditions described previously by Becker *et al.* (1993). The forward: 5'-ATACCACGCCAACGAAAAGAATGT-3' and reverse primers: 5'-CTATAGTACATATTGCATACCAT-3' were as described by Becker *et al.* (1993). The PCR amplification was carried out in a 25 µl volume containing 3 µl of DNA, 1 µl (10 µmol) of each forward and reverse primer, 12.5 µl of Master Mix (ThermoScientific Dream Taq™ Green PCR), and 7.5 µl distilled water.

Viral genomic DNA was subjected to thermal cycling that at 95 °C for 120 s, followed by 35 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 180 s, and a final extension at 72 °C for 300 s. PCR product (5 µl) was separated at 100 V for 20 min on a 1% agarose gel using the 100-bp DNA marker (ThermoScientific). PCR products were visualized in a gel documentation system (Clever Scientific, Ltd).

The amplified gC gene segments were sequenced using gene-specific primers by a commercial sequencing service (AB13730XL; Macrogen Inc., Seoul, Korea). Reference sequences and sequences of MDV gC under various accession numbers from different parts of the world were downloaded from <https://www.ncbi.nlm.nih.gov/nucleotide/> and aligned with the sequences of the present study using BIOEDIT (version 7.2.5). The phylogenetic trees were constructed using the neighbour-joining (N-J) tree method, and the validity of each branch was assessed using 1,000 bootstrap replications. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are presented in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura & Kumar, 2002). This analysis involved 37 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1547 positions in the final dataset. Evolutionary analyses were conducted in MEGA software (version 11.0.13) (Kumar *et al.*, 2016). The nucleotide identity percentage among the sequences was calculated using the sequence identity matrix tool in BIOEDIT (version 7.2.5).

The absolute MDV genome loads in splenocytes were quantified using real-time PCR with previously published primers specific to the *meq* gene of MDV using the extracted DNA (Abdul-Careem *et al.*, 2006). The *Meq* primer sequences were F-5'-GTCCCCCTCGATCTTTCTC-3' and R-5'-CGTCTGCTTCCTGCGTCTTC-3'. The DNA template was added to a total volume of 20 µl containing PCR buffer, oligonucleotide primers at 0.2 µM each of primer, and 2 µl of Fast SYBR™ Green Master Mix (Applied-Biosystems, Singapore). The chicken  $\beta$ -*actin* gene in each sample was amplified using a primer pair (F-5'-GAGAAATTGTGCGTGACATCA-3', R-5'-CCTGAACCTCTCATTGCCA-3'). To

describe the ratio between the two genes, the amplification of the *meq* gene was compared to the  $\beta$ -*actin* in each sample. The results were averaged across all samples tested in triplicate.

The gross examination findings and tumour lesions were documented using the criteria indicated by Burgess *et al.* (2001). The scoring of the lymphocytic infiltrations was conducted on formalin-fixed, paraffin-embedded (FFPE), 5- $\mu$ m thick sections of the liver, spleen, heart, and gonads, stained with Haematoxylin and Eosin. The following histologic scores were designed for this study as scattered lymphocytic infiltrations (+), moderate multifocal lymphoid cells clusters (++) and large multifocal to coalescing sheets of lymphocytes obliterating the tissue architecture (+++), as described by Kano *et al.* (2009). Each part was scored after a comparison with the established reference, and the overall scores were recorded.

The association of risk factors with MDV infection was analysed using a Chi-squared test. The relative fractions were expressed in percentages and 95% confidence intervals (95% CI) using GraphPad Prism 8 for windows 10 (GraphPad Software Inc., La Jolla, CA). Adjusted odds ratios related to dichotomous and polychotomous variables were assessed using Binary Logistic Regression Model using Minitab® 21.3.1 (64-bit). An independent *t*-test was used to analyse the difference between the mean viral loads of birds revealing gross tumorous lesions and those that did not. Spearman's ranked correlation was used to evaluate the correlation between the viral loads of the spleen samples and histological lymphocytic infiltration scores in the tissues. Statistical significance was defined at  $P < 0.05$ .

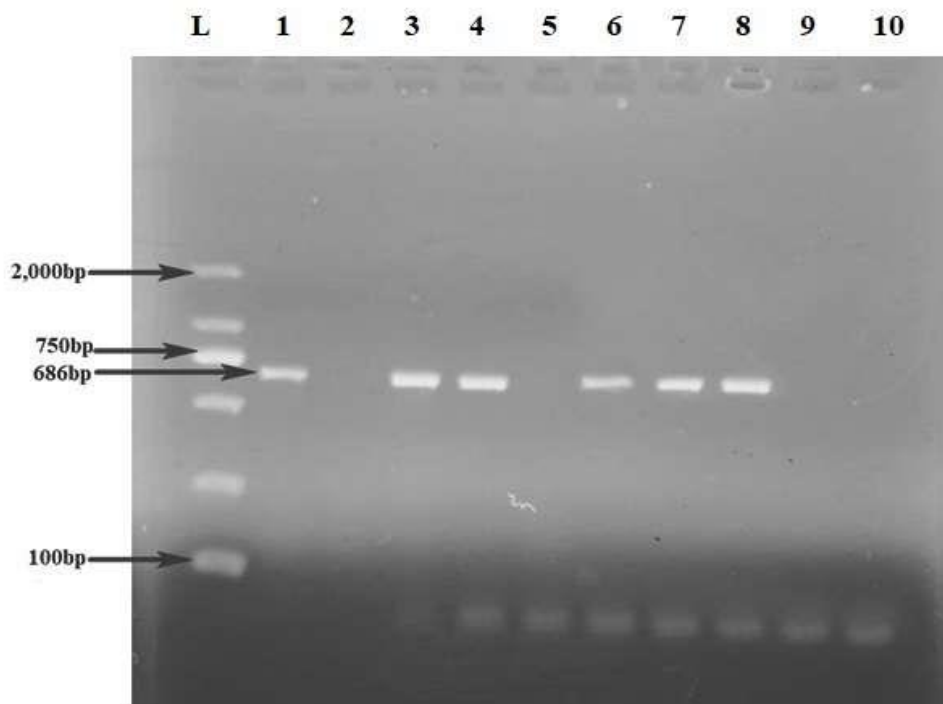
## Results

An overall 13.96% (95% CI; 19.80–9.64) presence of MDV infection based on gross morphology and 18.43% (95% CI; 24.75–13.43) with PCR was recorded in the chickens from the four districts of Rawalpindi division. The chi-squared test results did not reveal an association of disease positivity with the analysed risk factors ( $P > 0.05$ ). The birds with a vaccination history against MDV showed 23.48% fewer odds of infection than the unvaccinated individuals. Moreover, males showed almost 1.4-times more chance of infection than female birds. However, different backyard poultry breeds, including Aseel, Australorp, Fayoumi, and Rhode Island Red (RIR), had almost similar odds of infection with MDV. Table 1 shows the details of the infected and healthy birds with the associated factors.

**Table 1** Relationship of positivity of Marek's disease virus (MDV) in backyard poultry in Rawalpindi Division based on glycoprotein C-targeted polymerase chain reaction (PCR). The fractions are represented as percentages with 95% confidence interval. The adjusted odds ratios (OR) for dichotomous and polychotomous variables were calculated against a reference variable (\*).

Associated Factors		Uninfected percentage (95% CI)	Infected percentage (95% CI)	Adjusted OR (95% CI)
Vaccination Status	Unvaccinated	60.34% (67.22–53.03)	14.53% (20.44–10.11)	*
	Vaccinated	21.23% (27.8–15.88)	3.92% (7.86–1.91)	0.7652 (0.31–1.90)
Sex	Female	60.34% (67.22–53.03)	12.3% (17.91–8.26)	*
	Male	21.23% (27.8–15.88)	6.15% (10.67–3.47)	1.4211 (0.63–3.20)
Breed	Aseel	13.41% (19.18–9.18)	3.92% (7.86–1.91)	*
	Australorp	16.76% (22.92–12)	3.36% (7.12–1.55)	0.69 (0.20–2.31)
	Fayoumi	18.44% (24.76–13.44)	3.36% (7.12–1.55)	0.63 (0.19–2.09)
	Rhode Island Red	32.97% (40.15–26.5)	7.83% (12.7–4.72)	0.82 (0.29–2.26)

A positive PCR amplicon (686 bp) of the *gC* gene for MDV was identified using agarose gel electrophoresis. DNA extracted from different samples yielded a clear band in the gel electrophoresis after amplifying the target gene (Figure 2). The PCR amplified *gC* gene sequences were submitted to the GenBank database under accession numbers MN923520.1, MN923519.1, MN923518.1, MN923517.1, MN727368.1, MN727367.1, MN727366.1, and MN727365.1.



**Figure 2** Polymerase chain reaction amplification and ethidium bromide staining of Marek's disease virus *gC* gene (686 bp): Lane 1; positive control. Lane 3, 4, 6, 7, and 8; positive samples. Lane 10; negative control and L; DNA marker

The nucleotide sequence identity matrix revealed maximum similarity of MN727366.1 of 47.3% each with virulent strain virus isolates, KX290013.1, KU744561.1, KU744561.1, JX844666.1 (China) and EF523390.1 (USA). Isolate MN727365.1 showed 47.2% similarity each with isolates KX290013.1, KU744561.1, KU744561.1, JX844666.1 (China), and EF523390.1 (USA). Moreover, the isolate MN727368.1 had 47.1% similarity with KX290013.1, KU744561.1, KU744561.1, JX844666.1 (China), and EF523390.1 (USA). The isolates, MN727367.1, MN923520.1, and MN923519.1 showed 45.8%, 46.2%, and 46.2% similarity with JX533448.1, the mildly virulent Egyptian isolate. The phylogenetic analysis of nucleotide sequences revealed the three major clades. Isolates with accession numbers MN923518.1, MN923517.1, MN727368.1, MN727366.1, and MN727365.1 were placed with virulent US isolate (EF523390.1). MN727367.1, MN923520.1, and MN923519.1 were placed with mildly virulent vaccine strain virus CVI988 from the USA (DQ530348.1). The phylogenetic analysis of nucleotide sequences is shown in Figure 3.

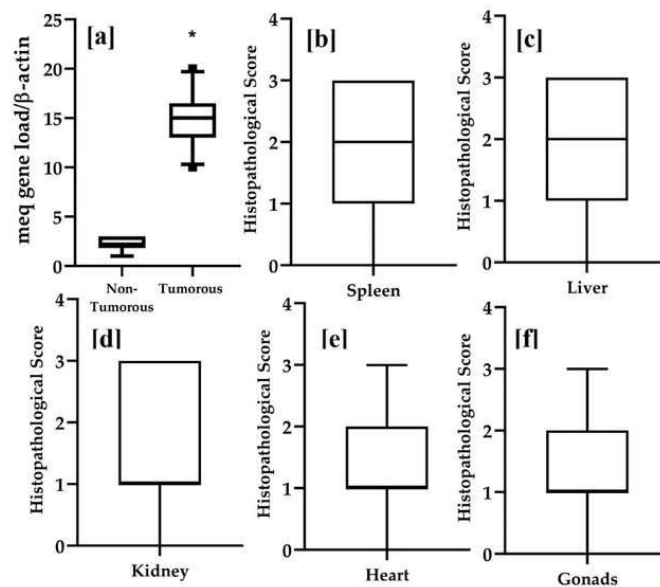






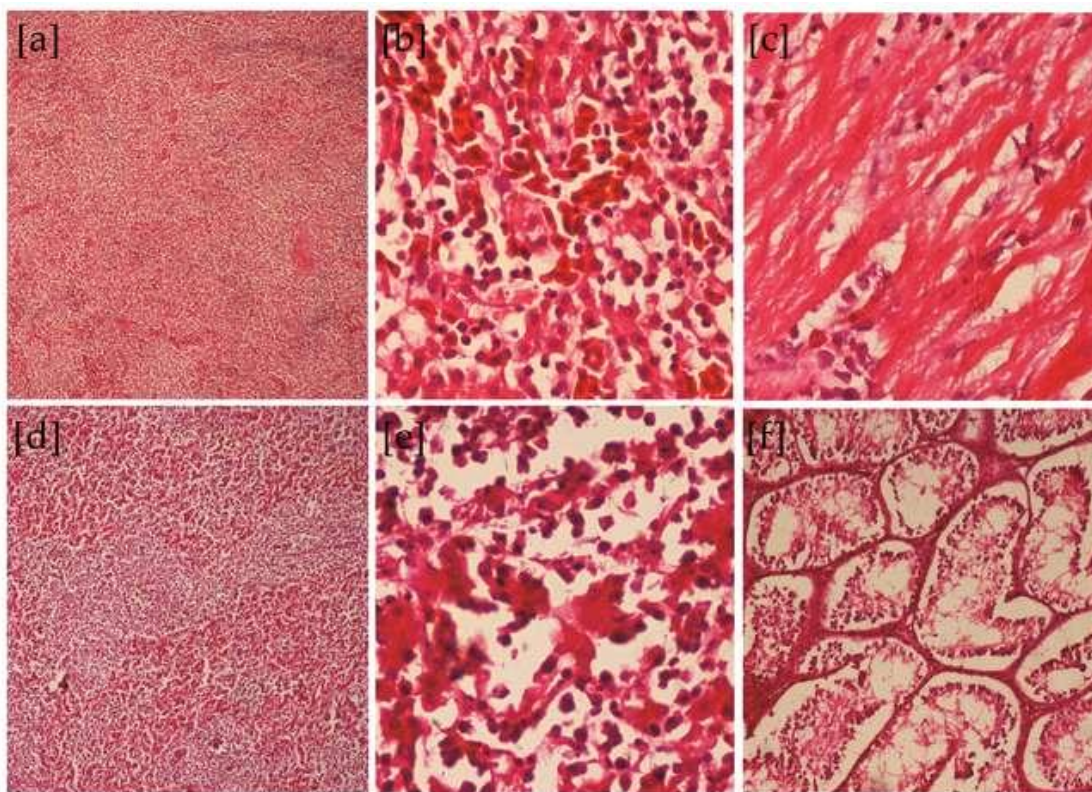
**Figure 4** (a) Affected bird showing cachexia. (b) The liver is diffusely enlarged with round borders. (c) Multifocal, pleomorphic lymphomas in the liver. (d) Swollen spleen with multifocal tumours. (e) Healthy liver and spleen of adult bird (f) Splenomegaly and hepatomegaly in birds affected with Marek's disease virus (MDV)

Total genomic DNA was isolated from the splenocytes of chickens, and MDV genome loads were quantified using real-time PCR. The MDV viral loads ranged between 10 and 20 copies of *meq/β-actin* in fresh spleens of the 25 tumorous chickens. The mean viral load quantified was 14.72 copies of *meq/β-actin* (95% CI; 13.69–15.75). The 5% and 95% percentile individuals lay at 10.3 and 19.7 copies of *meq/β-actin*, respectively. In the eight non-tumorous chickens, including the PCR-positive cases, MDV loads were at very low levels, revealing marked differences between tumorous and non-tumorous spleen samples. Figure 5 shows the *meq* gene load in the spleen tissues of both tumorous and non-tumorous individuals, along with the infiltrating lymphocytic score in the visceral organs of affected birds.



**Figure 5** Box and whiskers plots (a) Comparison of Marek's disease virus (MDV) *meq* gene load in the spleen tissues of birds with and without tumours. (b) (c) (d) (e), and (f) show histopathological scores of the spleen, liver, kidney, heart, and gonads, respectively.

A histopathological examination of the visceral organs revealed lymphoproliferative abnormalities in the heart, liver, spleen, kidney, and gonads. These lymphoproliferative changes varied from mild (+) and moderate (++) to severe (+++). Figure 6 describes histopathological lesions of varying severity in the various visceral organs of infected birds. Lymphoid tissue entirely replaced the parenchyma in several of the spleen tissues. There was a marked uniformity in the size of the neoplastic lymphocytes, ranging from tiny to medium. These lymphocytes were replacing segments of the parenchyma in the liver and spleen that ranged from multifocal to dispersed. The standard consistency of the organ was disrupted due to the presence of pleomorphic lymphoblastic and lymphocytic cells, comprising the majority of the cells in the organ. Median histopathological scores of spleen tissues were moderate (++) . Moreover, 95 percentile of individuals lay in severe (+++) histopathological scores (Figure 5b). In the liver, there was unequivocal evidence of both focal and diffuse pleomorphic cell infiltration. These cells were a combination of lymphoblasts, lymphocytes of small to medium size, plasma cells, and macrophages. Additionally, some macrophages with a few plasma cells were present. The infiltrating lymphoblasts were found to have a substantial number of mitotic figures. There was cellular infiltration of lymphocytes with replacement of normal parenchyma. Perivascular lymphoblast infiltration was also observed. Median histological scores of liver tissue were moderate (++) ; 95 percentile individuals revealed severe (+++) changes in the hepatic parenchyma. In heart tissue, observed median histological lymphocytic infiltration score was mild (+), (5 and 95 percentile of individuals lay between normal to moderate (++) scores). Gonads revealed median or mild (+) histological changes (5 and 95 percentile individuals shown normal and severe (+++) histological scores, respectively). Individual details of infected chickens are described in Table 2.



**Figure 6** Micrographs of Haematoxylin & Eosin-stained sections of the severely infiltrated (a & b) spleen (c) heart (d & e) liver, and (f) testis



**Table 2** Breed, vaccination histories, viral load, and histopathological score data of the 33 chickens confirmed with Marek's disease virus (MDV) using *gC* polymerase chain reaction (PCR). The lesion scores are described as usual (-), mild (+), moderate (++) and severe (+++).

Sample ID	Breed	Vaccination status	Sex	Absolute Viral Load	Histological Scores				
					Heart	Spleen	Liver	Kidney	Gonads
119	RIR	Unvaccinated	Female	1	-	-	-	-	-
21	Aseel	Unvaccinated	Female	2	-	-	-	-	+
97	Aseel	Unvaccinated	Female	2	+	+	-	-	-
15	RIR	Unvaccinated	Male	3	-	+	-	+	-
120	Aseel	Unvaccinated	Female	3	-	+	-	-	-
87	Fayoumi	Unvaccinated	Male	2	+	+	-	-	+
79	RIR	Unvaccinated	Female	2	+	+	+	-	-
166	Australorp	Unvaccinated	Male	3	+	+	+	-	+
16	RIR	Unvaccinated	Female	10	+	++	++	+	++
9	Aseel	Unvaccinated	Female	11	++	+	++	+	++
37	Fayoumi	Unvaccinated	Female	12	+	++	++	++	++
150	RIR	Unvaccinated	Male	12	+	+++	+	+	+
12	Fayoumi	Vaccinated	Male	13	+	++	+++	+	++
138	Aseel	Unvaccinated	Female	13	+	++	+	+	++
100	Australorp	Unvaccinated	Female	13	+++	++	++	+	++
40	Fayoumi	Vaccinated	Male	13	++	++	+++	+++	+
144	RIR	Unvaccinated	Female	13	+++	++	++	+	+++
163	RIR	Unvaccinated	Female	14	+++	+++	+++	+	+
73	RIR	Unvaccinated	Female	14	+	++	+	+++	++
173	Aseel	Unvaccinated	Male	14	+++	+++	++	+	+
133	Australorp	Unvaccinated	Female	15	+	+++	++	+++	+
27	RIR	Unvaccinated	Male	15	+	++	++	+++	++
161	RIR	Vaccinated	Female	15	+	+++	+	++	+
22	Fayoumi	Unvaccinated	Female	15	++	++	+++	++	+
169	Australorp	Unvaccinated	Female	15	++	+++	++	+++	+
82	RIR	Vaccinated	Male	16	+++	+++	+++	+++	++
153	Aseel	Unvaccinated	Female	16	+	+++	++	+	+++
51	Australorp	Vaccinated	Female	17	+++	+++	+++	+++	++
52	RIR	Unvaccinated	Female	17	+++	++	++	+++	++
111	Australorp	Vaccinated	Female	18	++	+++	+++	+	+
76	RIR	Unvaccinated	Female	18	+	+++	+++	++	+++
59	RIR	Vaccinated	Male	19	++	+++	++	++	+++
35	Fayoumi	Unvaccinated	Male	20	++	+++	+++	+++	+++

Spearman's ranked correlation regarding the quantification of MDV viral load (ratio of *meq/β-actin* copies) revealed a strong correlation with histological scores of various organs ( $P < 0.001$ ). The highest correlation was observed with the spleen (88%), followed by the kidney (77%), liver (82%), gonads (71%), and heart (61%). Table 3 shows the correlation matrix of the histological scores of various organs and viral loads in the spleen.

**Table 3** Matrix representing the correlation viral load (ratio of *meq/β-actin* copies) with the histological scores of lymphocytic infiltrations in the tissues of visceral organs (Spearman's ranked correlation calculated  $R^2$  for each variable).

	Viral load	Heart	Spleen	Liver	Kidney	Gonads	P-value
Viral load	-	0.61	0.88	0.82	0.77	0.71	
Heart	0.61	-	0.55	0.68	0.42	0.4	
Spleen	0.88	0.55	-	0.71	0.63	0.5	<0.001
Liver	0.82	0.68	0.71	-	0.61	0.57	
Kidney	0.77	0.42	0.63	0.61	-	0.45	
Gonads	0.71	0.4	0.5	0.57	0.45	-	

## Discussion

MDV has become more virulent over the last few decades, which has invited researchers globally to develop vaccines with many recent viral isolates. Although there are a substantial number of underlying connected elements, the cause of the continual increase in virulence is not known precisely (Jarosinski *et al.*, 2006). Lower levels of immune titre can result from a sub-adequate dose of vaccines, as compared to the recommended dosage. This could have been done for the sake of economic benefit, or it could have resulted from improper storage and handling (Witter, 1997). There are several different processes that might be due to mistakes while handling vaccinations, such as combining antibiotics with vaccines or performing the thawing process incorrectly. The higher occurrence rates can also be caused by other factors, including weak biosecurity (Dunn & Gimeno, 2013). The overall 13.96% (95% CI; 19.80–9.64) presence of MDV infection based on gross morphology and 18.43% (95% CI; 24.75–13.43) using PCR was recorded in chickens from the four districts of Rawalpindi division. This higher prevalence rate in the present study might be attributed to the fact that the sampling was done from the area where the field veterinarians were suspecting incidence of MDV in the backyard poultry.

It is well established that several structural proteins found in MDV are substantially involved in these processes. MDV glycoproteins found in the virus's envelope play a role in virus attachment and entrance or in virus maturation, emigration, and direct cell-to-cell dissemination, which have not been identified (Elliott *et al.*, 1995). *In vitro* and *in vivo* examinations of gC-like proteins, which are found in alpha herpes viruses and are the critical components of the viral envelope, reveal that these proteins have various roles in both conditions. In a broad sense, gC orthologues play an integral role in the first interaction between the virion and the cell by facilitating the attachment of the free virus to heparin- and chondroitin-like glycosaminoglycans present on the surface of the plasma membrane (Roizman & Knipe, 2001). In addition to their capacities to facilitate viral attachment and release, gC homologs are thought to be able to escape the immune system. It is believed that these functions are mediated by complement–component C3 binding, which then blocks the component's activity (Huemer *et al.*, 1993; Huemer *et al.*, 1995), in addition to playing a part in the process of leukocyte migration mediated by chemokines (González-Motos *et al.*, 2017). Several studies have demonstrated the effectiveness of PCR amplification of the gC gene for diagnosing MDV. One study, for example, used a PCR-based method to differentiate virulent from avirulent MDV strains based on gC gene amplification (Jarosinski & Osterrieder, 2012).

The phylogenetic analysis of gC gene nucleotide sequences revealed that present study isolates with accession numbers MN923518.1, MN923517.1, MN727368.1, MN727366.1, and MN727365.1 were placed with the virulent US isolate. MN727367.1, MN923520.1, and MN923519.1 were placed with the mildly virulent vaccine strain virus, CVI988, from the USA. The spleen tissue revealed higher viral load quantitation in the samples infected with the virulent MDV strain. In contrast, the birds infected with mild strain had substantially lower viral loads in the spleen tissue. Moreover, the birds also revealed marked differences in the gross and histopathological changes between both strains. The findings of phylogenetic analysis also concur with the *meq* gene-based quantitation of the virus in the splenic tissue. Similarly, Spearman's ranked correlation revealed a strong correlation (88%) of viral load with histological lymphocytic infiltration scores in the spleen ( $P < 0.001$ ).

The real-time PCR is a reliable technique to assess the severity of an MDV infection and the potential for developing MD lymphoma by measuring the *meq* gene load in infected tissues (Abdul-Careem *et al.*, 2006). The lesions in the present study were well-matched to those described by Hayajenh *et al.* (2021), Zelnik *et al.* (2004), and Fodor *et al.* (2001). Vieira-Pinto *et al.* (2003) described

that histopathology could be used to confirm the presence of MD. Neoplastic foci and pleomorphic cells were found during the histological analysis of liver and spleen tissues for the subclinical type of MD. There was evidence of lymphomatous lesions in certain areas containing lymphoblasts and lymphocytes. These lesions were usually elongated and ranged in size from small to medium. Although the tumour microenvironment is the same across organs, the extent of organ involvement can often vary (Stamilla *et al.*, 2020; Khan *et al.*, 2021). Wen *et al.* (2018) identified the presence of MD in the nucleus of neoplastic lymphoid cells found in the liver. Zelnik *et al.* (2004) reported the incidence of MD in vaccinated birds. It was observed by Khan *et al.* (2021) that infected liver cells proliferated around the small blood vesicles, and the lesions in the tumour consisted of proliferated tumour cells.

The capacity of the host to endure minimal detrimental consequences as a result of infection is what is meant by the term "tolerance," which refers to the term "genetic resistance to infection" (Råberg *et al.*, 2007; Jovanović *et al.*, 2009). Disease resistance mechanisms are complicated and incompletely understood at the molecular level. Genetic resistance to infectious diseases is controlled by a number of variables, including both biological and environmental factors (Zekarias *et al.*, 2002; Zeleke *et al.*, 2005). The majority of research that has been conducted on disease resistance mechanisms has focused on investigating the function of innate immune responses. This is because it is generally acknowledged that innate immune responses are the factors that determine whether or not an individual is resistant or susceptible to infectious pathogens (Glass *et al.*, 2012; Iwasaki & Medzhitov, 2015). There is a correlation between the major histocompatibility complex, also known as MHC haplotypes, and the variation in disease susceptibility and resistance (Janeway, 2001; Leveque *et al.*, 2003; Kapczynski *et al.*, 2013).

To a great extent, antigen processing and presentation rely on the MHC molecules. Because each gene has several variations in the population, the MHC can process and display any antigens that might exist (Janeway Jr *et al.*, 2001). The MHC genotype limits T-cell antigen recognition. The specific T-cells might not recognize the antigen because of the defect in the MHC molecule. Consequently, the MHC molecules are responsible for controlling the antigen specificity of T-cells (Janeway Jr *et al.*, 2001). According to specific reports, there is a connection between the MHC haplotypes and the varying predispositions of chickens to infectious diseases (Goto *et al.*, 2009). However, in the present study, different backyard poultry breeds, including Aseel, Australorp, Fayoumi, and Rhode Island Red had almost similar odds of infection with MDV.

## Conclusion

In conclusion, it is suggested that virulent and mild pathotypes of MDV-1 strains are prevalent in the backyard poultry of the Rawalpindi division, which is a continuous source of threat for commercial poultry in the study area.

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## Authors' contribution

MA and MURK conceived the idea and facilitated the execution of research activities. MA, MURK, GS, and MHS performed the sampling and testing. MA and MURK facilitated in the statistical analysis, manuscript write-up and reviewing it.

## Conflict of Interest

The authors declare that there is no conflict of interest related to this article.

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