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Th1 and Th2 Cytokines Activity during Transformation and Lymphoma Formation Stage in Chicken Naturally Infected with Marek's disease virus.

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Abstract: *Marek's disease virus (MDV), a highly transmissible cell-associated neuropathic oncogenic alphaherpes virus affecting poultry health, resulting in considerable economic losses in poultry industry worldwide.Until now MDV still emerging and re emerging causing great economic losses in chicken despite of intensive vaccination and management policy used in poultry farms. However, cytokines and its role in MD pathogenesis and immunity had been described by some workers under certain experimental conditions by using different MDV strains challenge, they need to be more clarified during the more progressive lymphoma transformation stage. The present study aimed to examine the transcriptional profiling of a panel of cytokines genes in the splenic tissues of special broiler Japanese chickens (70-80 days old) contracted natural infection with MDV despite of intensive care and vaccination policy adopted by HVT and CVI988/Rispene. SYBR Green-based, real-time (RT)-PCR protocol was used to quantitate cytokine mRNA in freshly collected spleen tissue of MDV infected and control chicken. Changes in the levels of spleen interleukins (IL) as IL-6, IL-10, IL-18 and IL-12P35, IL-4, interferon-gamma (IFN-γ) and inducible nitric oxide synthase (iNOS) mRNA was determined. Relative Messenger RNA (mRNA) expression levels of the above mentioned genes, and β-actin as a reference gene, were achieved. The results of quantitative real-time PCR (qPCR) assays using* $\Delta\Delta$Ct method *revealed significant up regulation in the expression levels of IL-6, IL-10, IL-18, and IL-12P35). The changes in the mRNA levels of IL-4, IFN-γ and inducible nitric oxide synthesase (iNOS) were minimal and not significant in comparison to those in uninfected age-matched control chicken. In conclusion, these data strongly support the hypothesis that pro-inflammatory responses, including high levels of Th2 cytokines as IL-10 and other interleukins as IL-6, IL-18 and IL12-P35, may play a major role during MDV lymphoma transformation stage induced by MDV strain of high virulence. These cytokines may be involved in maintenance of MDV infection and lymphoma formation. On the other hand, Th1 cytokines as IFN-γ, and iNOS had no or minimal role in induction of MDV-specific immune response during MDV lymphoma transformation stage.*

Keywords*: MDV; lymphoma, Cytokine; Th1; Th2; q-PCR.*

I. Introduction

Marek's disease virus (MDV) is a highly transmissible cell-associated neuropathic oncogenic alphaherpes virus (Lee *et al.*, 2000; Tulman *et al.*, 2000) affecting poultry health worldwide, characterized by T-cell lymphomas in peripheral nerves and visceral organs (Ding *et al.*, 2007) resulting in considerable economic losses.Three serotypes of MDV have been classified; MDV-1 which is the pathogenic one for chickens, causing marek's disease (MD), is classified as gallid herpes virus II. The second serotype; MDV-2, is gallid herpes virus III and the third and last one is a MDV-3 (as HVT) as meleagrid herpes virus. The second and third one are non-oncogenic and have been commonly utilized in vaccines against MD (Alexander *et al.*, 2003; van Regenmortel *et al.*, 2000). Unexpected MD outbreaks and greater MD-associated losses in vaccinated flocks had consequently increased (Witter, 1997) despite of intensive vaccination policy used. Emergence of hypervirulent strains (Burgess *et al.*, 2004; Schumacher *et al.*, 2002), with pronounced increase in virulence (Witter, 1997), suggested to be the cause of vaccination failure.

MDV genome encodes more than 200 genes (Tian *et al.*, 2011).Meq gene, one of the most important MDV proteins, present only in MDV-1 strains. It is highly expressed in MDV-1-transformed cell lines and tumor samples (Jones *et al.*, 1992; Lupiani *et al.*, 2004). Recently, meq gene has attracted attention as a possible cause

for increased oncogenicity (Shamblin *et al.*, 2004; Tian *et al.*, 2011; Wozniakowski *et al.*, 2010), although many other genes also play important roles in the development of lymphomas (Jarosinski *et al.*, 2006).
Pathogenesis of MD starting from virus entry followed by various sequential events involve lytic infection of B-cells then latent infection and oncogenic transformation of T-cells in lymphoid tissues, peripheral nerves and visceral organs (Alexander *et al.*, 2003; Baaten *et al.*, 2004; Baigent and Davison, 2004; Calnek, 2001). Non-specific (innate) and specific (adaptive) host responses are elicited in response to MDV infection. Innate defense response emerge soon after infection, whereas adaptive immune responses are usually detectable later around 5 to 7 dpi and include the development of MDV-specific antibodies and cytotoxic T lymphocytes (CTL). Cytokines are involved in the orchestration of both arms of the immune system, in addition to the above responses (Davison and Kaiser, 2004).

Production of cytokines and the potential role of cytokines in immunity against MD have been mentioned under experimental condition (Jarosinski *et al.*, 2005; Kaiser *et al.*, 2003; Xing and Schat, 2000a). Many experiments in susceptible and resistant birds were done to see the role of this cytokines. There is up regulation of mRNA level of some cytokines as IFN-γ mRNA in different organs, such as the spleen (Abdul-Careem *et al.*, 2007; Heidari *et al.*, 2008; Xing and Schat, 2000b) and feather follicle epithelium (FFE) (Abdul-Careem *et al.*, 2008b; Abdul-Careem *et al.*, 2008c; Abdul-Careem *et al.*, 2009b).

Pathogenesis of MD, as well as protective vaccination against MD, is associated with up-regulation of a group of interleukins such as IL18, IL6, IL10, IL8 and IL1β (Abdul-Careem *et al.*, 2007). This up-regulation was detected in different organs such as spleen, bursa of Fabricius, lung and FFE (Abdul-Careem *et al.*, 2008a; Abdul-Careem *et al.*, 2008b; Abdul-Careem *et al.*, 2009a; Abdul-Careem *et al.*, 2007; Heidari *et al.*, 2008; Parvizi *et al.*, 2009).On the opposite side, there is down-regulation of type 1 interferon, particularly IFN-α (Ambagala and Cohen, 2007; Quere *et al.*, 2005).In addition to morphopathological examination, and PCR identification, we aimed in the present study to investigate the effect of naturally emerged Japanese MDV strains of high virulence (data under publication), on the expression of someT-helper 2 (Th 2) and (Th1) cytokines to see their roles on the pathogenesis and development of visceral lymphoma in vaccinated chickens.

II. Material And Methods

1.1. Sample collection and histopathological examination:

Samples were collected during 2016 from 30 special broiler flocks (70-80 days old). They were all vaccinated by HVT& CVI988 /Rispens and brought to (Pathology laboratory, Faculty of Applied Biological Science, Gifu University, Japan) suspected to be affected by oncogenic viruses for necropsy and diagnosis. Five to ten chickens (from each flock) developed progressive tumors in different visceral organs were sacrificed according to slaughter house guidelines in Japan and brought immediately (half an hour after slaughtering) to (Pathology laboratory, Faculty of Applied Biological Science, Gifu University, Japan) for necropsy and diagnosis. Chickens were immediately subjected to careful postmortem (PM) examination and all PM findings were recorded. Tissue specimens were immediately collected from all tumor bearing organs including, liver, kidney, spleen, proventriculus, heart, intestine and sciatic nerves. A set of tissue specimens were immediately fixed in 10% neutral buffered formalin (average period of fixation was 3–4 days at room temperature) for histopthological examinations. Another set were collected and kept frozen at -20 C until used for RNA & DNA extraction.

Fixed specimens were routinely processed through dehydration in ascending grades of ethanol and then cleared in xylene and embedded in paraffin blocks. Paraffin sections were prepared and some were stained with hematoxylin & eosin for histopatholgical examinations.

1.2. DNA& RNA extraction from collected samples:

Total DNA& RNA extraction from nerve and visceral tumor tissues including liver, spleen, kidney, proventriculus were done according to (Qiagen, AllPrep® DNA/RNA Mini kit, Germany) manufacturer's instructions. On the same side, Total DNA& RNA were extracted from nerve and the same visceral organ tissues of uninfected age-matched controlled birds reared under the same condition from healthy farms. Total RNA samples were reverse-transcribed by using (QuantiTect ®Reverse Transcription Kit, Germany), according to the manufacturer's instructions. cDNA from spleen was used for specific PCR amplification of cytokines; IL-4 ,IL-6, IL-10 , IL-12 p35, IL-18, IFN-γ, iNOS, and β-actin acted as the reference gene using specifc primers previously published (Abdul-Careem *et al.*, 2006; Abdul-Careem *et al.*, 2007). The primers were synthesized by Sigma–Aldrich-Japan. Spleen was used to obtain target cDNA, as it was known that spleen harbor all stages of the MDV life cycle (Baigent and Davison, 2004) and plays an important role in elicitation of immune responses.

1.3. Conventional PCR:

Conventional PCR for the detection of MDV-meq gene from spleen and other visceral organs DNA preparations using previously published specific primer (Hassanin *et al.*, 2013) was done for initial MDV screening. The PCR amplification was performed in a 50 µl volume containing 2 µl of DNA, 4 µl of dNTP, 1 µl (10 µMol) of each primer, 5 µl 10x Ex Taq buffer, 0.25 µl Ex Taq polymerase (Takara, Kyoto, Japan), and 36.75 µl distilled water. The thermal cycling was 95 °C for 2 min followed by 40 cycles at 95 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. All PCR reactions were carried out using the (Takara Thermal cycler, Japan). The PCR products were visualized and photographed in a 1% agarose gel containing 0.5 mg/ml ethidium bromide on a Gel Documentation System.

1.4. Preparation of constructs as standards:

The average C_T (cycle threshold) was calculated for β-actin with each primer used in this study. The efficiencies of the target and reference genes are similar, and the $\Delta\Delta Ct$ calculation for the relative quantification of target used when the absolute value of the slope is close to zero.

The standard curves construction for target genes including IL-4, IL-6, IL-10, IL-12 p35, IL-18, IFN-γ, iNOS, and β-actin as a reference gene have been generated using 10-fold serial dilutions (10^{-1} to 10^{-9}) of the relevant cDNA preparations and assayed in duplicate or triplicate.

The PCR efficiency (*E*) values for standard curves ranged from 1.99 to 2.01. *R*2 (coefficient of regression.) values for the standard curve reactions ranged from 0.991 to 0.999, with the majority of values above 0.995 in all examined genes used in this study.

1.5. Real-time PCR and amplification protocol:

Relative mRNA expression levels of IL-4, IL-6, IL-10, IL-12 p35, IL-18, IFN-γ, iNOS, and β-actin as a reference gene, were achieved using Takara, Thermal Cycler Dice™ Real Time System Single Software with software version 4.02. All reactions were performed by Syper ® premix Ex Taq ™ (Tli RNaseH plus), Takara Cclontech by using a 25 µl volume in each reaction capillary. For quantification of the cytokines; 2 µl cDNA was added before capillaries were capped, centrifuged, and placed in the Light Cycler sample carousel. Cycling parameters were as follows: 3 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at the annealing temperature (ranged from 55°C to 60°C according to each primer, and 20 s at 72°C, dissociation curve analysis was performed immediately after q- PCR amplification of each target with the reference gene with continuous fluorescence acquisition.

III. Results

1.6. Gross Pathological Findings:

Grossly, the peripheral nerves of few cases of the affected birds showed swelling, edema, loss of striation and discoloration. On the other side, many cases showed no macroscopic changes in peripheral nerves.

Gross picture of visceral organs showed severe enlargement in one or all of the following organs, liver, spleen, lung, proventriculus, intestine and kidneys. The lesion distribution ranged from focal to multifocal, white raised foci on the surface. Similar nodular gross, deeply sited within the parenchyma of the organs cut-section to few solitary nodules was observed. The size of the nodules ranged from pin point foci to large nodules of 1-2 cm in diameter (Fig.1. A& B). Some cases showed only diffuse enlargement without nodular focal lesions.

Histopathological examination of peripheral nerves revealed, predominance of type C nerve lesions represented by marked vacoulation and demylination of most of the nerve tissue with very scarce small lymphocytic infiltrations especially in perineuronal nerve sheath (Fig.1. C& D).

Histopathological examination of visceral organs revealed that, lymphoproliverative changes in the visceral organs including Liver, spleen, kidney, lung, proventriculus, and intestine were more sever in intensity and distributions. Visceral organs showed highly progressive infiltration of neoplastic lymphoblast with high degree of pleomorphism, many mitotic figures were detected in the infiltrating lymphoblast (Fig.2. A, B, C, and D). The parenchyma of some severely affected organs was completely replaced by lymphoblastic elements. Detailed description of histopathological examination was described (data under publication).

1.7. Detection of MDV genome by PCR:

The results of PCR amplification of representative visceral tumor and peripheral nerve tissue samples, representing the thirty vaccinated chicken flocks, were positive for t Meq gene (Fig.3). CVI988 MDV strain was used in this study as positive controls. Uninfected control chickens had no MDV DNA and all were negative by *PCR.*

1.8. Quantification of cytokine mRNA expression:

Splenic tissues from MDV vaccinated chickens naturally infected by field isolates of MDV of highly virulent strain (data under publication), demonstrated different patterns of expression of certain cytokines in response to MDV infection. Messenger RNA (mRNA) expression of target genes (IL-4, IL-6, IL-10, IL-12 p35 subunit, IL-18, IFN-γ and iNOS) was expressed relatively to β-actin gene expression, which served as the reference, in the same sample preparation. mRNA was isolated and the levels in infected and control samples were quantified using relative quantitative PCR assay (qPCR) using $\Delta\Delta$Ct method. Statistical significance is evaluated using unpaired T-test for comparison between controlled uninfected matched age and infected birds. Comparisons were considered significant at ($P \leq 0.05$), β-actin was amplified in all reactions at approximately the same level for both uninfected and infected groups of chickens.

All examined cytokines were detected in the control group by different levels of each one. These levels were up regulated in most of infected examined birds, but not all this upregulation is significant by statistical analysis. Infected chickens had significantly higher IL-6, IL-10, IL-12 p35 and IL- 18 mRNA expression compared to controlled chickens (Fig.4. A, B, C, and D), ($P \leq 0.05$).

However, the difference in IL-4 mRNA expression in the spleen of MDV-infected and uninfected chickens was not significantly different and had *p* values ($P > 0.05$) (Fig.5. E). In addition, the expression of IFN-γ, iNOS was minimal and not significantly different among both groups ($P > 0.05$) (Fig.5. F and G).

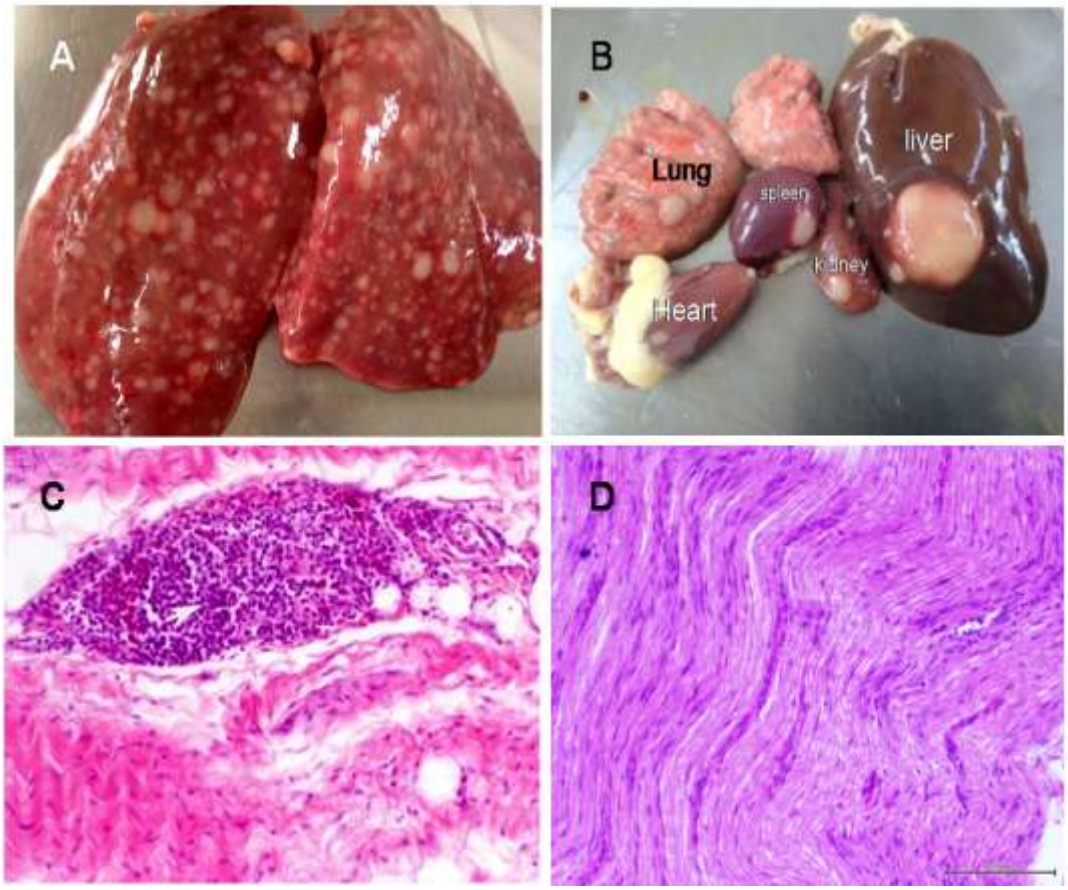

Fig.1. (**A–B**): Gross lesion in poultry. (**A**) Showing numerous white neoplastic nodules of variable size throughout the enlarged liver lobes. **(B**) Visceral organs (liver, spleen, lung, kidney and heart) notice white nodules of variable size in all organs except heart. (**C**) Peripheral nerve of lymphoma bearing chicken showing accumulation of inflammatory lymphocytes in nerve sheath (white arrow) H&E, X400.(**D**) Peripheral nerve showing mild lymphocytic cellular infiltration in-between nerve axons (type C MDV nerve lesions), H&E, Bar=50μm.

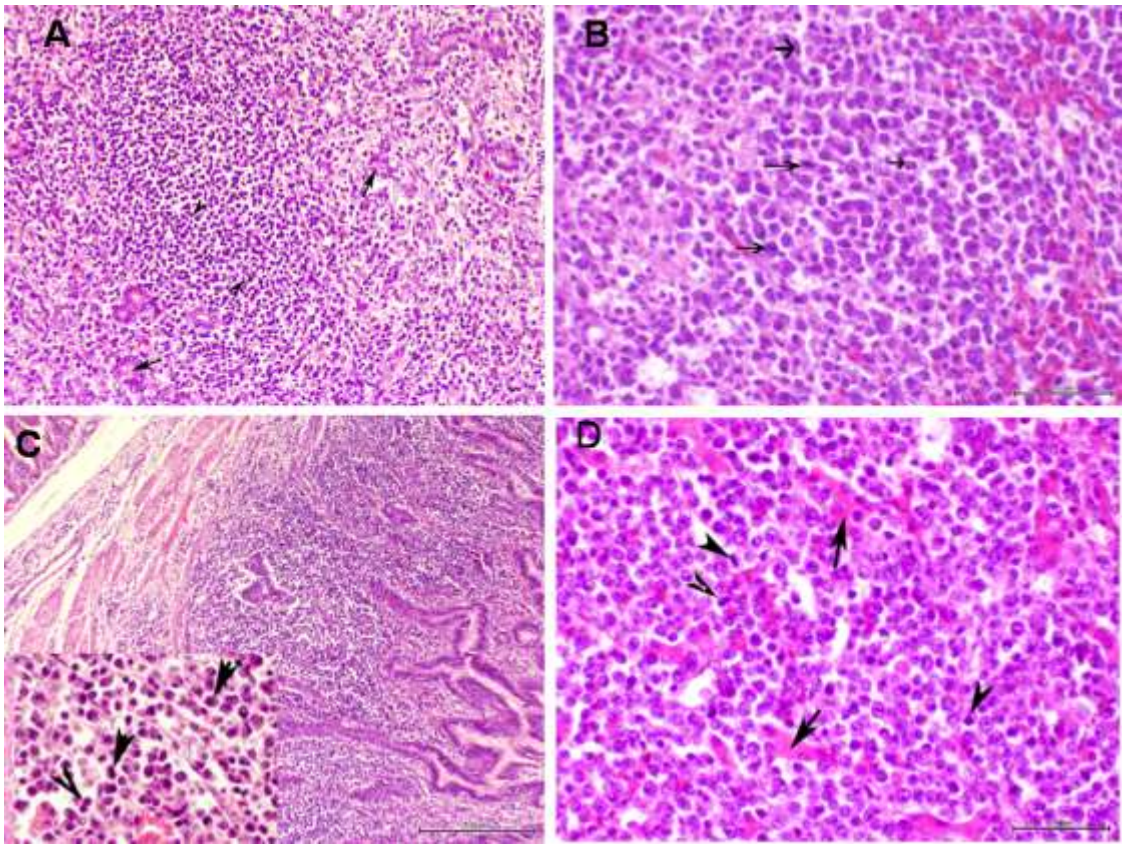

Fig.2. (**A**) Kidney showing marked lymphoblastic cellular infiltration (head arrows) among remnant of degenerated and necrosed renal tubules (arrows) H & E, Bar=50µm. (**B**) Spleen showing polymorphic, neoplastic lymphoblastic cellular infiltration, notice numerous mitotic figure (arrow) H & E, Bar=20µm. (**C)** Proventriculus of diseased chicken showing heavy infiltration of mucosal and submucosal layer by lymphoblastic cells .H & E, Bar=100µm, the inset is higher magnification of infiltrating cell, notice multiple mitotic figures (arrow head)**. (D)** liver showing diffuse heavy infiltration of pleomorphic lymphoblatic cells of highly mitotic activities (head arrows) with scarcely distributed, remnant of degenerated hepatocytes (arrows).H & E , Bar=20µm.

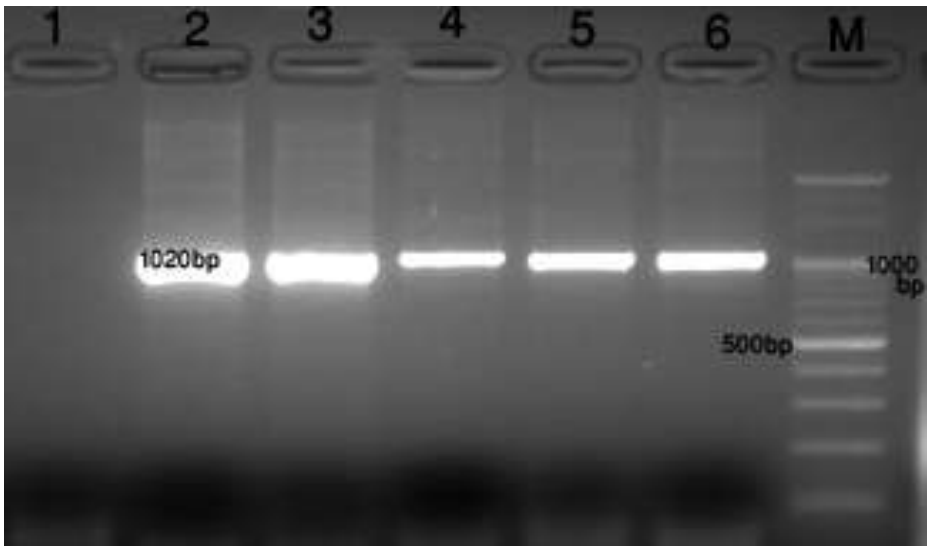

Fig.3. Results of PCR amplification of MDV Meq gene of examined chickens using specific primer. Lane1: negative control sample; Lane2: positive control sample; Lanes 3–6: representing positive chicken samples from different flocks showing amplification of a 1020 bp product; Latest lane (M): DNA ladder marker (1kbp marker).

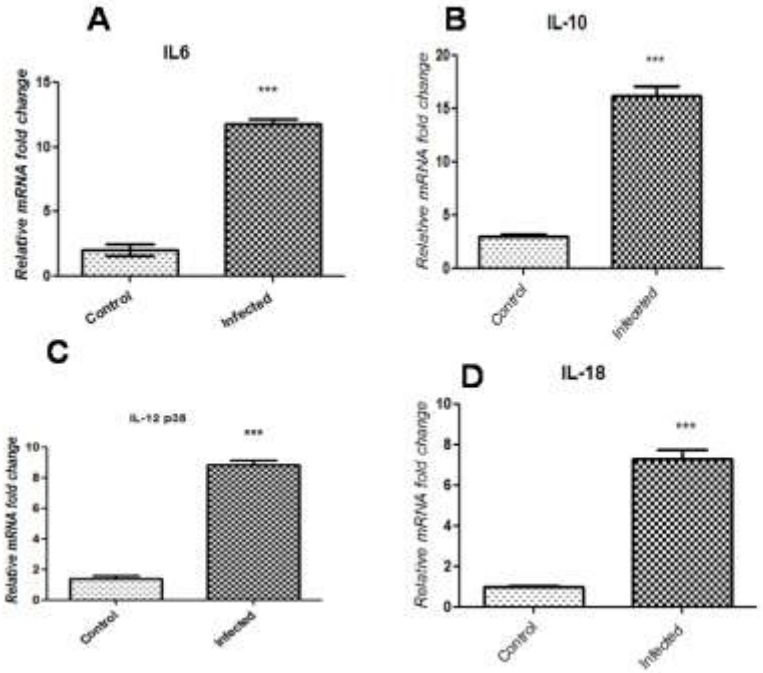

Fig.4. Cytokine mRNA expressions in spleen tissues of MDV infected chickens with developed progressive visceral lymphomas compared to age-matched, uninfected control chickens. Target gene expression is presented relative to β-actin expression and normalized to a calibrator. (A) IL-6, (B) IL-10,(C) IL-12 p35, and (D) IL-18. The difference between both groups was assessed by analysis of variance followed by unpaired T- test and comparisons were considered significant at $P \leq 0.05$. The value P***<0.001 is highly significant. Vertical bars represent the standard error.

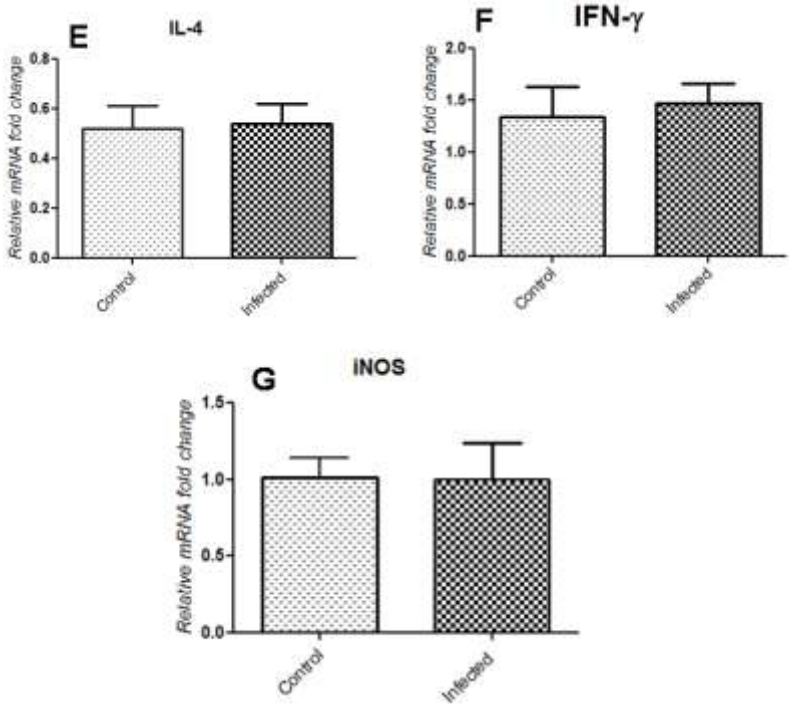

Fig.5. Cytokine mRNA expressions in spleen tissues of MDV infected chickens with developed progressive visceral lymphomas compared to age-matched, uninfected control chickens.. Target gene expression is presented relative to β-actin expression and normalized to a calibrator. (**E**) IL-4, (F) IFN-γ and (G) iNOS. The difference between both groups was assessed by analysis of variance followed by unpaired T- test and comparisons were considered non significant at ($P > 0.05$).Vertical bars represent the standard error.

IV. Discussion

Emergence of more virulent strains of MDV is a significant problem for the poultry industry and need more studies to catch up the causes of increased pathogenecity and virulence of this oncogenic virus.

Cytokines have been implicated in pathogenesis of several herpesviruses and they play important roles in the course of MDV pathogenesis. Virus replication is associated with expression of pro-inflammatory cytokines in different tissues mainly spleen, bursa of fabricious and brain, and there is a correlation between viral virulence and the amount of expressed cytokines (Aravalli *et al.*, 2005; Jarosinski *et al.*, 2005).

In this study, we sought to determine the effect of newly emerged Japanese MDV field isolates of highly virulence (data under publication) on the expression of some Th1 and Th2 cytokines on chickens naturally infected by these circulating field strain with development of progressive multi-focal lymphoma in different visceral organs. To find the role of these expressed cytokines on pathogenesis or protection against this virus, the transcription of certain cytokines genes from spleen tissues from control and MDV-infected chickens were investigated in this study.

Recognized progressive multi-tumor formation on different visceral organs including liver, lung, kidney, spleen and proventriculus had been detected during postmortem examination with minimal observed gross lesions were detected in peripheral nerves of this chickens except little swelling and yellowish discoloration in some birds as observed before in various studies (Davison and Nair, 2004; Saif *et al.*, 2003).

Chicken cytokines were measured by different methods as TaqMan real-time PCR assays (Jarosinski *et al.*, 2005; Kaiser *et al.*, 2003), and SYBR Green-based method (Abdul-Careem *et al.*, 2007; Heidari *et al.*, 2008; Sadeyen *et al.*, 2004). The SYBR Green-based method is more sensitive and able to quantify as low as 40–150 chicken cytokine transcripts (Abdul-Careem *et al.*, 2006).

In our study, MDV infection was associated with enhanced expression of IL-6, IL-10 and IL-18 genes (Abdul-Careem *et al.*, 2008a; Abdul-Careem *et al.*, 2008b; Abdul-Careem *et al.*, 2009a; Abdul-Careem *et al.*, 2007; Heidari *et al.*, 2008; Parvizi *et al.*, 2009). In contrary to this results, the previous genes were down regulated during latency stage of MDV infection (Heidari *et al.*, 2008).

IL-6, a pro-inflammatory cytokine has been shown previously to be up regulated in MD susceptible chicken lines and that elevated levels may be due to increased pathology in this lines (Kaiser *et al.*, 2003) and also it is up regulated in spleens and brains of chickens infected with MDV (Jarosinski *et al.*, 2005; Kaiser *et al.*, 2003; Xing and Schat, 2000a).

IL-18, another pro-inflammatory cytokine, is correlated directly with IL-6 expression, as it was known that, IL-18 is involved in induction of IL-6 in mammals (Netea *et al.*, 2000), so significant IL-6 expression is associated along with significant IL-18 expression. Significant lower expression of these two cytokines was observed in vaccinated protected group compared to unvaccinated controls. It was hypothesized that, lower expression of these two cytokines may be associated with protection conferred by vaccines (Abdul-Careem *et al.*, 2007).

Transcriptional activity of IL-6 and IL-18 were enhanced in our study and this results were in consistent with earlier study (Abdul-Careem *et al.*, 2007; Heidari *et al.*, 2008) as this cytokines were detected in MD susceptible birds and not in MD resistant ones. This results in our opinion speculated that, this cytokines may play important role in driving immune response to MDV infection to form lymphoma in susceptible lines.

IL-10 was highly expressed in infected chickens in comparison to all examined cytokines (up to 13 fold difference), and this observation is correlated to its function as B cells activator, and its important role in the pathogenesis of MDV as enhanced expression of IL-10 may be a strategy exploited by MDV to evade the host immune system (Marshall *et al.*, 2003). Over expression of IL-10 may explain the increased severity of pathological pictures induced by MDV of high virulence. IL-10 leads to down regulation of immune response (Th1 responses) to intracellular pathogens (Stober *et al.*, 2005) and its production is associated also with parasites persistence (Belkaid *et al.*, 2001; Belkaid *et al.*, 2002) and vaccine failure (Stober *et al.*, 2005).

IFN-γ expression is inversely proportional to IL-10 production, as IL-10 inhibits IFN-γ production in chickens (Rothwell *et al.*, 2004). So it is logically to found lower expression of IFN-γ and progressive lymphoma formation due to MDV, as the main function of IFN-γ as antiviral activity (Djeraba *et al.*, 2000; Lee, 1979; Xing and Schat, 2000a) was inhibited by over production of IL-10 produced by regulatory T cells.

In another previous studies, a reduction in IFN-γ levels in blood, with reduction in macrophages number and activity has been reported during infection of genetically susceptible chickens by RB1B strain (vv+) of MDV (Quere *et al.*, 2005). This result is in agreement with our result of minimal IFN-γ expression.

In the current study, the changes in the mRNA levels of IFN-γ and inducible nitric oxide synthesase (iNOS) were minimal and not significant in comparison to those of the control uninfected age-matched birds and this results were in consistent with previous investigation (Abdul-Careem *et al.*, 2007).

The role of IL-12 p35 gene in the chicken immune system and pathogenesis of a viral infection in chicken is poorly understood and the first reports about its rule in MDV infection in chicken was mentioned by (Abdul-Careem *et al.*, 2006). They reported that enhanced expression of IL-12p35 level in response to MDV infection

and its level peaked in infected chickens with neurologic manifestations. In our study, IL-12 p35 expression was significantly higher in- MDV-infected chickens with lymphoma formation compared to uninfected chickens.
In summary, we report here that MDV infection is associated with a significant increase Th-2 cytokines (IL-6, IL-10 IL-18) and IL12-P35 genes expression and these cytokines are involved in maintenance of MDV infection and lymphoma formation. Other cytokines include; IFN-γ and iNOS have minimal role in driving immune response during lymphoma transformation stage.

The expression pattern of IL-6, IL-10 IL-18 and IL12-P35 genes is up regulated following MDV infection then they down regulated during latency (Heidari *et al.*, 2008). In the present study we report that they up regulated again during MDV transformation and lymphoma stage.

In conclusion, these data strongly support the hypothesis that pro-inflammatory responses, including high levels of Th2 cytokines as (IL-10) and other interleukins as IL-6, IL-18 and IL12-P35, may play a major role during MDV lymphoma transformation stage induced by MDV strain of high virulence. These cytokines may be involved in maintenance of MDV infection and lymphoma formation. On the other hand, Th1 cytokines as (IFN-γ), and iNOS had no or minimal role in induction of MDV-specific immune response during MDV lymphoma transformation stage.

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