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Molecular and Immunopathological Investigations of Marek's Disease in HVT Vaccinated Chicken Flocks

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ABSTRACT

Background: The present investigation was carried out on poultry carcasses brought to the Department of Veterinary Pathology, LUVAS, Hisar from Herpesvirus of turkeys (HVT) vaccinated chicken flocks and suspected for viral neoplastic conditions on the basis of necropsy. Conventional and molecular techniques for the confirmation of the Marek's disease (MD) were studied. For differential diagnosis from other oncogenic viral infections immunohistochemistry as an important tool was investigated.

Methods: The conventional diagnosis of MD) was done on basis of clinical signs, gross lesions and histopathology of proliferate lesions; as lymphomatous lesions consist of pleomorphic lymphocytes. For confirmatory diagnosis molecular techniques were employed to detect MD virus specific Meq gene by Taqman probe real time PCR and nested PCR. For differential diagnosis of mixed oncogenic viral infections, immunohistochemistry (IHC) using B and T cell differentiation markers (CD79 alpha and CD3) was employed.

Result: Specific Meq gene by Taqman probe real time PCR was positive in 92.5% suspected cases; while nested PCR technique gave positivity in 55% cases. The immunohistochemical reactivity for CD3 gave positive reactivity in the cytoplasm and cell membrane and negative reaction for CD79 alpha in MD positive cases, while in cases with mixed infections of MDV and other viral neoplastic conditions, cells showed positive reactivity to both CD3 and CD79 alpha in neoplastic proliferates.

Key words: CD79 alpha and CD3, Immunohistochemistry, Marek's disease, Nested PCR, Real time PCR.

INTRODUCTION

Marek's disease virus serotype 1 (MDV1) is an oncogenic DNA virus which belongs to the family *Herpesviridae* subfamily *Alphaherpesvirinae* and genus Mardivirus causing the Marek's disease (MD) of poultry. Major broader clinical signs in MD are immunosuppression, polyneuritis and lymphoproliferative nodules formation in the visceral and cutaneous tissues (Witter and Schat, 2003). The traditional diagnosis of MD is based on clinical signs and gross pathology. Histopathological examination of proliferate lesions is also very important in diagnosing MD; as lymphomatous lesions consist of pleomorphic lymphocytes. Even with the intensive vaccination practice, outbreaks of MDV associated lymphocytic tumors and paralysis was reported from India (Kamaldeep, 2007) and other countries resulting in great economic losses. Additionally, the increasing virulence and the immunosuppressive effect of MDV makes it a threat to broiler industry as it has been reported to increase their susceptibility to *Escherichia coli* and infectious bronchitis (IB) infection (Islam *et al*., 2002).

Prompt and differential disease diagnosis therefore plays a key role to minimise the losses by enabling early adaptation of proper control and preventive measures. The principal methods to confirm the avian oncogenic diseases are isolation of the virus, demonstration of viral DNA or antigens in tissues and detection of antibody (Schat and Nair, 2008). PCR appears to be a method of choice for the diagnosis of avian oncogenic viruses and IHC can be used in differential diagnosis of these neoplastic conditions (Gopal *et al*., 2012). Therefore; for optimizing production, it is mandatory to use the correct pathological, microbiological and other diagnostic techniques, which may be helpful to check the disease occurrence and minimize the losses in production.

In the present study, we report the diagnostic investigation of MD in HVT vaccinated organized poultry farms of Haryana using immunopathohistochemical and molecular techniques.

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MATERIALS AND METHODS

The present investigation was carried out on 40 cases received from HVT vaccinated organized poultry farms suspected for viral neoplastic conditions on the basis of gross and histopathological lesions which were brought to the Department of Veterinary Pathology, LUVAS, Hisar during the period of 6 months (from August 2018 to January 2019).

History and collection of samples

The detailed identification of all the carcasses was recorded as per requisition form. Representative tissue samples from abnormal nodular growths were collected during necropsy in 10% neutral buffered formalin for histopathological and immunohistochemical studies and in separate sterile vials without preservative which were stored at -20°C for molecular studies.

Molecular studies

Molecular diagnosis and characterization studies were carried out to detect MD virus specific Meq gene by Taqman probe real time PCR and nested PCR in all the cases which revealed lymphomas on the basis of gross and histopathological examination.

TaqMan probe real time PCR for target Meq gene of Marek's disease virus

Total genomic DNA was extracted from liver tumor tissue samples (400 mg) using commercially available genomic extraction DNA Mini Kit (DNeasy® Blood and Tissue Kit, Invitrogen) as per manufacturer's tissue protocol. For the identification of MDV specific target gene (Meq), a Taqman probe real time PCR assay was performed using real time PCR Applied Biosystems (Step one plus) for data acquisition and analysis. The reaction for the meq gene was carried out in triplicate for each sample. Oligonucleotide primers and probes used in the PCR are described in Table 1. The results for TaqMan probe real time PCR were analyzed on the basis of their cycle threshold (CT) values.

Nested PCR for target Meq gene of Marek's disease virus

All the samples screened by TaqMan probe real time PCR were also subjected for nested PCR for qualitative analysis. The first round of PCR was performed with the primer set M-S and M-AS to amplify a 1,062-bp Meq gene fragments (Lee *et al*., 2000). The sequences of MDV specific primers (Murata *et al*., 2007) used are shown in the Table 2. The second round of PCR was performed using primer set of MR-S and MR-AS to amplify a 583-bp Meq gene (Chang *et al*., 2002). The expected size of PCR product was estimated by comparison with that of standard DNA ladder.

Immunohistochemical studies

Immunohistochemical staining using primary antibodies for B and T cell differentiation markers i.e. CD79 alpha (anti-chicken Invitrogen; CAT No.MA5-13212) and CD3 (anti-chicken Invitrogen CAT No. MA5-28699) was done in formalin fixed tumor tissues as per standard protocol (Ramos-Vara *et al*., 2014). Antigen retrieval was performed by microwave irradiation with 0.01 M citric buffer, pH 6.0, for 10×2 minutes. Optimal concentration of each primary antibody was determined by standard protocol. Immunohistochemical staining was accessed as negative and positive groups. Brick red or brown red colour in membrane and cytoplasm of tumor cells was taken as positive.

RESULTS AND DISCUSSION

Pathological studies revealed mortality due to neoplastic disease conditions in 12.15% cases; with 7.79% in layers, 2.12% in broilers and 2.15% in dual purpose chicken breeds affecting mainly more than 18 weeks age group. Most of the affected chickens showed nonspecific clinical signs in the form of decreased feed intake, depression, emaciation and paleness. In contrary paralytic symptoms of the legs were not present and no lesions were detected in nerves, bursa of Fabricius, skin and eyes in our study.

Similar findings have also been observed by other researchers (Sani *et al*., 2017) who reported that the overall prevalence of avian neoplastic diseases was 7.58% with

Table 1: Oligonucleotide primers and probes used in TaqMan probe real time PCR for Meq gene.

Target sequence	Primer/probe name	Primer sequence (5′-3′)	Primer location	Amplicon size (bp)
MDV-1 *meq* gene (Jones *et al*., 1992)	Meq forward	GGTCTGGTGGTTTCCAGGTGA	1341-1361	73
	Meq reverse	GCATAGACGATGTGCTGCTGA	1413-1393	
	Meq probe	AGACCCTGATGATCCGCATTGCGACT (5′FAM label, 3′TAMRA label)	1366-1391	

Table 2: Sequences of MDV specific Meq primers used for nested PCR amplification.

Primers	Sequences	Location ofprimers	Expected sizes of amplified fragment
M-S	5′-ATGTCTCAGGAGCCAGAGCCGGCGCT-3′	1-26	1062
M-AS	5′-GGGGCATAGACGATGTGCTGCTGAG-3′	1038-1162 (1218-1242)	
MR-S	5′-TGTTCGGGATCCTCGGTAAGA-3′	347-367	583
MR-AS	5′-AGTTGGCTTGTCATGAGCCAG-3′	909-929 (1089-1109)	

overall prevalence of MD as 6.25%. They further added that the average age of poultry affected was 20.17 (SD=±2.6) weeks for MD and 41.43 (SD=±15.4) weeks for AL with weight loss (55.98%), ruffled feathers (47.01%), diarrhea (39.79%) and leg paralysis (17.52%) as the most reported clinical signs. In agreement to our findings clinical signs of poor growth and production have also been reported by various researchers (Das *et al*., 2018). The existence of genetic resistance to MD among chickens and its influence on the outcome of MDV infection has been recognized since long (Payne and Venugopal, 2000). Similar to our observations, it has been observed that the mortality in broiler breeder was more in females than males (Eid *et al*., 2019); which indicate that the outcome of infection is influenced by sex, as females are usually more susceptible to the development of tumors (Payne and Venugopal, 2000). The occurrence of MD in vaccinated flocks has also been reported by earlier workers (Kamaldeep *et al*., 2007). Exposures to chicken anaemia virus infection, emergence of hyper virulent strains (Raja *et al*., 2009), with further increase in the virulence of field strain suggested to be the cause of vaccination failure and pathological picture change of this neoplastic disease (Gong *et al*., 2013). Incidence of MD in commercial layer flocks despite vaccination have also been related to vaccine failure consequent to prolonged exposure to high levels of trace elements, particularly lead and cadmium, leading to immunosuppression (Muniyellappa *et al*., 2015).

Grossly, nodular enlargements in liver, spleen, lungs, kidneys, proventriculus, intestine and ovaries were histopathologicaly confirmed by the proliferation and infiltration of pleomorphic lymphomatous cells, suggestive of MD. Microscopic lesions in MD are consisting of diffusely proliferating small-to- medium lymphocytes, lymphoblasts and activated and primitive reticulum cells. Published data suggests that all tumors with uniform lymphoblast population can be of MD or LL (Payne *et al*., 1976). In such cases, confirmatory diagnosis can be made based on immunocyto/ histochemistry (Meq, IgM) and ruling-out virus infection (MDV/ALV) by PCR. Five cases in present study also revealed aggregates of uniform sized large lymphoid cells (lymphoblasts) suggestive of avian lymphoid leucosis (LL) indicating mixed infections of MD with ALV. Mixed infections need differentiation by a simultaneous immunoreactivity with both Meq and IgM for MD and LL specific immuno markers respectively. Histopathological evidence did not support the presence of reticuloendotheliosis (RE) type lesions in any case.

The results for TaqMan probe real time PCR indicated that 92.5% cases gave positive expression for MDV specific Meq gene; while 3 cases were undetermined. Amplification plot depicting CT (cycle threshold) values has been presented in Fig 1. The CT value ranged between14.69 to 35.9. 72.9% cases revealed CT value ≥29 indicating strong positive, while 27.1% cases showed CT value between >29-37 indicating moderate positive result.

Results of nested PCR confirmed the presence of 583 bp product for MDV specific Meq gene which is depicted in Fig 2. Out of total 40 cases, 22 cases showed positive expression of Meq gene of Marek's disease virus. On comparison, the sensitivity of TaqMan probe real time PCR was 92.5% in detecting MDV specific target gene (Meq) while it was only 55% in case of nested PCR. The findings were more or less in agreement to those of other workers (Krol *et al*., 2007). Marek's disease (MD) is caused by a cell-associated herpes virus and the genetic material of MDV is a linear, double stranded DNA molecule containing about 80 specific genes of MDV which encode peptides that have an essential significance for oncogenicity and pathogenicity. Main oncogenes are the products of gene Meq, pp38 and 132bp sequence. It was found that high loads of MDV DNA and expression of the MDV oncogene Meq were specific for MDV-induced lymphomas and valid criteria for MD diagnosis (Gimeno *et al*., 2005). Researchers also established that the Meq gene is necessary for the development of tumors

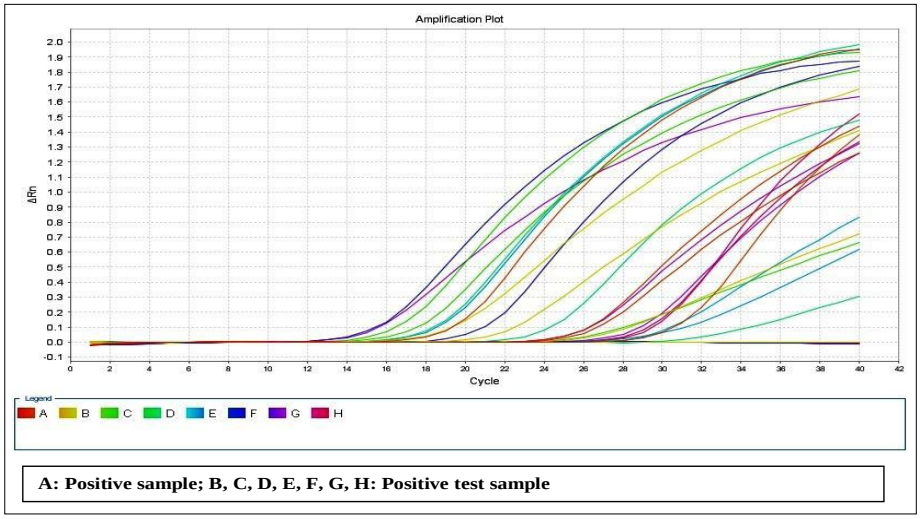

Fig 1: Amplification plot depicting CT values giving positive expression for MDV specific Meq gene in TaqMan probe real time PCR.

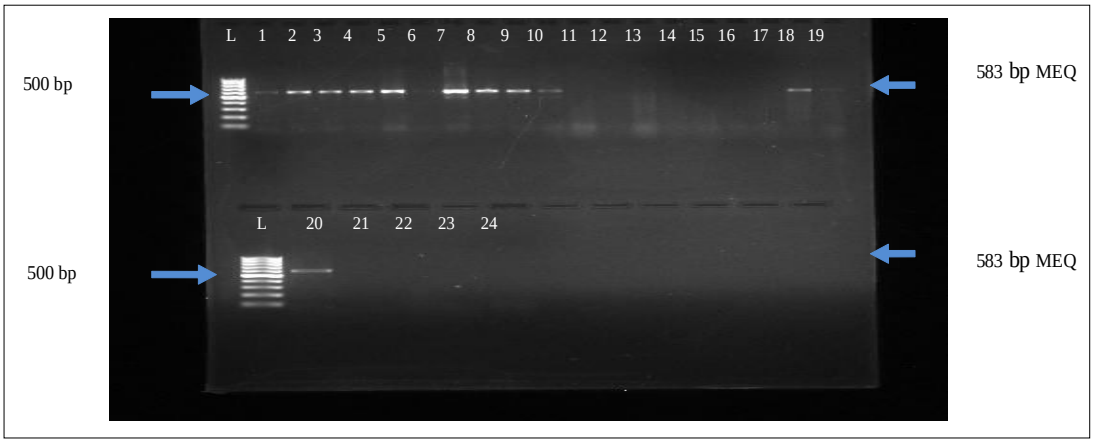

Fig 2: Resolution of nested PCR amplified product for Meq gene on 1.5% agarose gel.

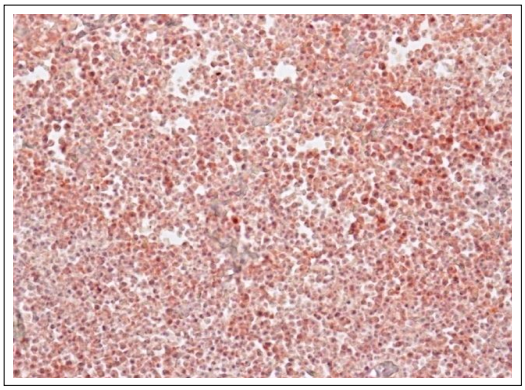

Fig 3: Positive membranous and cytoplasmic immunoreactivity to CD3 in the neoplastic lymphoid cells in Marek's disease affected liver tissue (IHC stain 200X).

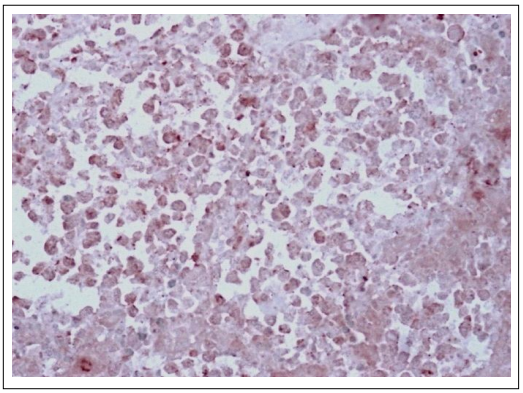

Fig 4: Positive membranous and cytoplasmic immunoreactivity to CD3 in few pleomorphic neoplastic cells (MD+LL infection) (IHC stain 400X).

and therefore is a key gene in the pathogenesis of MD (Lupiani *et al*., 2004). Molecular detection of MDV specific Meq gene DNA in tumors, especially neoplastic nodules consistent with gross and histopathological characteristics has been found to be reliable for MD diagnosis.

Real-time polymerase chain reaction (PCR) methods based on different detection systems can determine the MDV genome loads based on either relative (Yunis *et al*., 2004) or absolute quantification (Baigent *et al*., 2005). The importance of real time PCR is to precisely distinguish and measure specific nucleic acid sequences in a sample, even if there is only a very small amount of DNA. The sensitivity of the real-time assay was found to be 2.5–10 times more than that of conventional PCR. Results of present study also indicate more sensitivity of real time PCR than nested PCR for Marek's disease diagnosis which is in agreement to the findings of Dagher *et al*., (2004).

The result of immunohistochemical reactivity to B and T cell markers indicated that for CD3 antibody (T cell marker) was found to be positive in almost all MD positive cases with more than 90% cytoplasmic or membranous reactivity in neoplastic cells *i.e*. both small and large lymphocytes (Fig 3). In cases suspected of mixed infections, having both pleomorphic and uniform large lymphoblast cells, positive reactivity was observed in few pleomorphic neoplastic cells only (Fig 4). No immunoreactivity for CD79 alpha was observed in MD cases (Fig 5) but in few cases there was positive membranous and cytoplasmic immunoreactivity in few large lymphocytes cells particularly at perivascular locations. Mixed infection cases, revealed positive immunohistochemical reactivity to CD79 alpha marker in 20 to 50% neoplastic cells particularly in large uniform immature lymphoblast cells (Fig 6) indicating the presence of B cells in tumor tissue. Results therefore support the utility of immunohistochemical staining besides the histological appearance in differentiating MD from other neoplastic diseases.

These findings were similar with the results of other researchers (Mete *et al*., 2016 and Eid *et al*., 2019). Negative immunoreactivity for CD79 alpha antibodies in pleomorphic lymphoid cells indicates that B cells did not comprise the neoplastic lymphoma lesions in MD. However, mixed infection cases indicate presence of both T and B cells in proliferating lymphomas. The findings were similar with the results of some workers (Pejovic *et al*., 2007 and Haridy

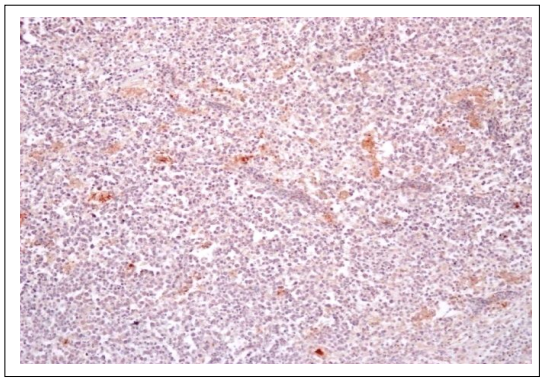

Fig 5: No immunoreactivity to CD79 alpha in neoplastic lymphoid cells in Marek's disease affected liver tissue. Few necrotic hepatocytes showing positive reddish brown immunostaining in the cytoplasm (IHC stain 200X).

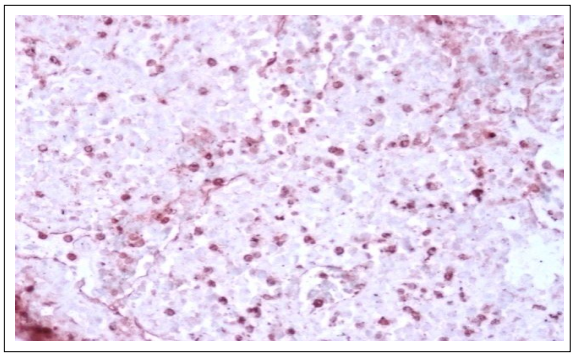

Fig 6: Positive membranous and cytoplasmic immunostaining to CD79 alpha in few neoplastic lymphoid cells (MD+LL mixed infection) (IHC stain 400X).

et al., 2018) who revealed negative immunoreactivity of CD79 alpha in liver, kidney and spleen lymphomas in chickens and for CD20 (B-cell marker) and Pax5 (B-cell transcription factor) in white silkie fowl, respectively. According to our findings we also conclude that the CD3 cell population was dominant in MDV lesions. However, there were a modest percentage of CD79 alpha positive cells in the liver tumors, located perivascularly. In Marek's disease B cells are infected with MDV during the cytolytic phase of infection. Therefore, B-cells do play an important role in MDV pathogenesis and their presence in MDV lymphomas was also previously reported (Payne *et al*., 1976). The results could be helpful in diagnosing mixed infections of MD and LL as well. Reports on multiple oncogenic virus infections have been described in literature and at times ALV and REV have been detected as contaminants in Marek's disease vaccines (Zavala and Cheng, 2006).

CONCLUSION

It may be concluded from the present findings that immunohistochemistry and molecular technique employing TaqMan probe real time PCR using Meq oncogene in tumor tissues in addition to conventional methods such as necropsy and histopathology could be useful for confirmatory diagnosis of poultry viral neoplastic infections which is still posing to be a major problem in vaccinated chicken flocks.

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Conflict of interest: None.

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