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Pathological and molecular diagnosis of Marek's Disease in desi birds

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Abstract
The objective of this study was to identify the visceral type of Marek's Disease in unvaccinated desi birds. A total of eight dead birds of age 13–15 weeks from a desi poultry flock were presented for necropsy to the Department of Veterinary Pathology, NTR CVSc, Gannavaram. The visceral form of MD was diagnosed by gross, cytological and histological examinations. On external examination the birds were emaciated, dehydrated, with pale and anaemic combs and wattles. Grossly, visceral organs (liver, spleen, kidneys, testes, heart, lungs and proventriculus) were enlarged with presence of focal to diffuse nodular growths. Cytological examination of impression smears collected from heart, liver, and spleen during necropsy revealed a mixed population of pleomorphic lymphoid cells. Histopathology of these affected organs revealed diffuse infiltration of pleomorphic lymphoblasts, small and medium-sized lymphocytes, a few plasma cells, and macrophages. Molecular diagnosis of MD was performed by polymerase chain reaction (PCR) amplification of the vv pathotype of Marek's Disease Virus in suspected birds by targeting 434bp fragment of the LTR region.

Keywords: Visceral form, nodular growths, cytological examination, polymerase chain reaction

Introduction
Neoplastic diseases in chicken are often caused by viruses and have a major economic impact (Nair *et al*., 2013) [16]. Marek's Disease Virus (MDV), Avian Leukosis Virus (ALV), and Reticuloendotheliosis virus (REV) are the three major oncogenic pathogens. MD is a re-emerging viral disease that affects chicken and is caused by the Marek's disease virus (Buckmaster *et al*., 1988 and Payne & Rennie, 1976) [4, 19], which is a linear ds DNA virus, is an *Alphaherpes virus* with a linear double-stranded DNA genome. It is a member of the subfamily *Alphaherpesvirinae*, genus *Mardivirus* and the species, *Gallid herpesvirus* 2 (Davison, 2010) [6]. Based on their pathogenicity and virulence, MDV is classified into three serotypes: 1, 2, and 3 (OIE, 2010) [17], among which only serotype 1 is capable of inducing tumours (Davison, 2010) [6]. Based on their virulence, serotype 1 strains are further divided into pathotypes, which are often referred to as mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) MDV strains (Witter *et al*., 2005) [28]. Infected dander is easily spread in farm premises and inhaled by healthy birds (Shambu *et al*., 2012) [23]. Generally, MD can occur at any age, beginning at 3–4 weeks of age or older, with occasional outbreaks reported in adult chickens (Zhuang *et al*., 2015) [30]. The incubation period ranges from 3-4 weeks to several months.
The disease is manifested in various forms *viz*; a) Neurological form – Acute infiltration of the central nervous system and peripheral nervous system causes paralysis of the legs and wings; b) Visceral form – Tumours in the heart, ovaries, testis, muscles, and lungs in both males and females; and c) Cutaneous form – Tumours of feather follicles. Infections caused by MDV1 are associated with lymphoproliferative lesions, which can include nervous affection, paralysis, bursal atrophy, parenchymal neoplastic cellular infiltrations, pleomorphic lymphomas, and neoplastic cell infiltrations in several visceral organs, nerves, muscles, and the skin (OIE, 2018) [18]. The present study was undertaken to determine histopathological changes in MD affected organs and to identify the Marek's disease viral antigen by PCR as well.

Materials and Methods
Post-mortem and sample collection
A total of eight dead birds of age 13–15 weeks from a desi poultry flock were presented for

necropsy to the Department of Veterinary Pathology, NTR CVSc, Gannavaram with a history of reduced growth rate, depression, anorexia, and death. Impression smears were collected from the heart, liver, and spleen and stained with Leishman's stain for cytological study (Luna, 1968) [14]. Suspected tissue samples from different organs, i.e., liver, spleen, kidney, testes, proventriculus, and lungs were collected in 10% neutral buffered formalin for histopathological examination. For molecular diagnosis, tissues from the liver and spleen were collected from suspected birds in sterile tubes and stored at -20 °C.

Histopathology

The tissues collected from organs showing gross lesions were subjected to histopathological examination. The tissues were dehydrated, cleared, and embedded in paraffin by routine manual processing. They were cut into 4-5 microns with a Rotary microtome and stained with Haematoxylin and Eosin routinely. These were mounted with DPX mountant solution and covered with cover slips for microscopical examination (Luna, 1968) [14].

DNA isolation and PCR

DNA was isolated from suitable tissues by using the HipurA® Mammalian Genomic DNA Purification Kit and primers were used specifically targeting the LTR region of MDV for amplification of the vv pathotype of MDV. The primers used were i.e., F: 5' TACTTCCTATATAGATTGAGACGT 3' and R: 5' GAGATCCTCGTAAGGTGTAATATA 3' as described by Visnuvinayagam *et al*. (2019) [27]. The PCR conditions were standardised as per the details given in Table 1. Electrophoresis in a 1% agarose gel in 1 X TAE buffer (Thermoscientific) was used to detect the PCR product. The gel was visualized, and the results were documented in a gel documentation system (Biorad).

Table 1: PCR conditions for the amplification of Marek's disease virus

Steps		Temperature	Time
Initial denaturation		94 °C	4 min
34 cycles	Denaturation	94 °C	1 min
	Annealing	57 °C	1 min
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Hold		4 °C	10 min

Results

A total of eight dead birds of age 13–15 weeks from a desi poultry flock were presented for necropsy to the Department of Veterinary Pathology, NTR CVSc, Gannavaram with a history of reduced growth rate, depression, anorexia, and death. In addition, affected birds showed decreased egg production, with reduced size of eggs and mortality, causing severe economic losses.

Gross pathology

Grossly, the birds were emaciated, dehydrated, with pale and anaemic combs and wattles. On internal examination, the liver was severely enlarged and occupied the complete abdominal cavity with multiple, diffuse whitish nodules of varying sizes, from pinpoint foci to 1-2 cm in diameter. On cut section these nodules were firm, smooth and deeply seated in the parenchyma (Fig. 1). The spleen showed diffuse enlargement with variable sizes of greyish white neoplastic foci (Fig. 2). Kidneys were swollen and showed focal nodular growths (Fig. 3). Testes revealed unilateral marked hypertrophy and congestion (Fig. 4). The heart was enlarged with single or multiple nodular growths in the myocardium. The Lungs were enlarged because of tumorous growth, and the proventriculus was thickened with nodular growth. However, the brain or peripheral nerves were devoid of any appreciable gross lesions.

Cytological examination

Impression smears were collected from the organs showing the lymphoid growths and, on staining and examination, revealed a mixed population of pleomorphic small to large lymphocytes, lymphoblasts, and plasma cells in the heart, liver, and spleen (Fig. 5).

Histopathology

Histopathologically, the liver revealed focal to diffuse infiltration of pleomorphic lymphoblasts, small and medium-sized lymphocytes, a few plasma cells, and macrophages, leading to disruption of hepatic architecture, and a few mitotic figures were noticed in infiltrating lymphoblasts. Degeneration and necrosis of hepatocytes were also observed, along with the infiltration of lymphoid cells (Fig. 6). The spleen revealed pleomorphic infiltration of lymphoid cells which were mostly limited to perivascular areas (Fig. 7). The kidney revealed focal lymphoid cell proliferation displacing renal parenchyma and necrotic changes in adjacent renal tubules (Fig. 8). Numerous pleomorphic lymphoid infiltrations in the heart, lung, and submucosa of the proventriculus were also seen suggestive of MD.

Molecular diagnosis

In the present study, tissue samples of the liver and spleen that showed characteristic lesions of MD were utilised for molecular diagnosis of MDV. The DNA was extracted and amplified by PCR targeting the LTR region of the vv pathotype of MDV. Gel electrophoretic analysis of the PCR product revealed an amplified product of a 434 bp fragment of the LTR region, thus confirming the presence of MDV in the suspected tissue samples (Fig. 9).

Discussion

In the present study, clinical signs observed were reduced growth rates, inappetence, depression, inactivity, anorexia, and decreased egg production, with reduced size of eggs and mortality causing severe economic losses. These were in accordance with the reports Marek's disease by Sung (2002) [25], Kalyani *et al*. (2010) [11], Xu *et al*. (2011) [29], and Singh *et al*. (2012) [24]. Cytological examination of impression smears from the heart, liver, and spleen of the affected birds revealed a mixed population of pleomorphic small to large lymphocytes, lymphoblasts, and plasma cells suggestive of Marek's disease, which were also reported by Swathi *et al*. (2012) [26] and Reddy *et al*. (2021) [20]. On post-mortem examination, externally affected birds were emaciated, dehydrated, with pale and anaemic combs and wattles. These findings were in line with the reports of Santin *et al*. (2006) [21], Arulmozhi *et al*. (2011) [2], Musa (2013) [15], Sawale *et al*. (2014) [22], and Das *et al*. (2018) [8]. Internal examination of the birds revealed enlargement because of nodular growth in the visceral organs, i.e., liver, spleen, and proventriculus, which were akin to reports of Kamaldeep *et al*. (2007) [12], Jayalakshmi *et al*. (2016) [10], Balasubramaniam *et al*. (2017)

[3], Das *et al*. (2018) [8], and Abd-ellatief *et al*. (2018) [1], the kidney, lungs and heart showed lesions similar to the reports of Das *et al*. (2018) [8]. Histopathologically, focal to diffuse pleomorphic infiltration of lymphoid cells in the liver, spleen and kidney was also reported by Abd-ellatief *et al*. (2018) [1], Balasubramaniam *et al*. (2017) [3], Das *et al*. (2018) [8] and Chacon *et al*. (2019) [5]. Numerous pleomorphic lymphoid infiltrations observed in the heart, lung, and submucosa of the proventriculus were similar to the reports of Abd-ellatief *et al*. (2018) [1], Balasubramaniam *et al*. (2017) [3] and Lounas *et al*. (2021) [13]. The polymerase chain reaction (PCR) has emerged as an additional diagnostic tool offering serotype specificity (Davidson *et al*., 1995) [7] and it has the ability to differentiate between vaccinal and field strains of MDV (Handberg *et al*., 2001) [9]. Jayalakshmi *et al*. (2016) [10] and Visnuvinayagam *et al*. (2019) [27] confirmed the presence of MDV in the liver and spleen respectively by PCR amplification of the 434bp region by targeting the LTR region of the vv pathotype of MDV in MD suspected birds.

Figures

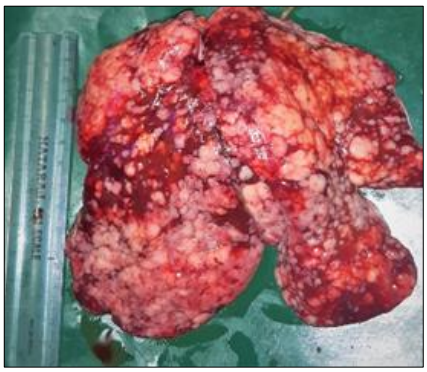

Fig 1: Liver - Enlargement with multi focal lymphoid tumours

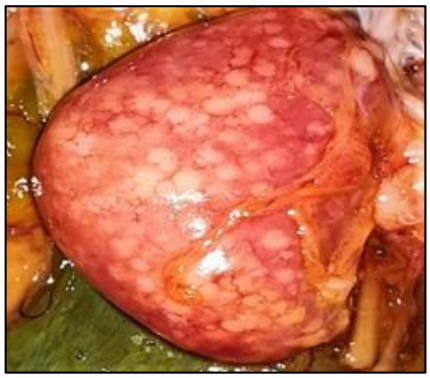

Fig 2: Spleen - diffuse enlargement with varied size neoplastic foci

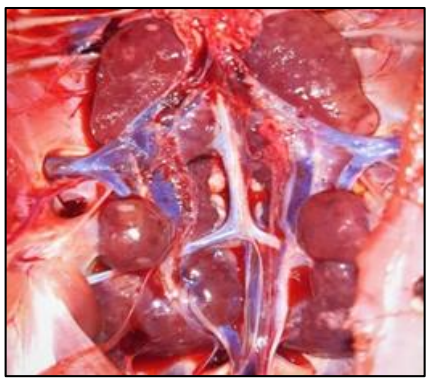

Fig 3: kidney - enlargement and focal nodular growth**.**

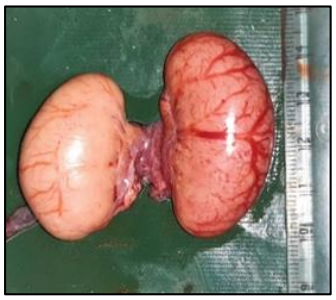

Fig 4: Testes - unilateral marked hypertrophy and congestion**.**

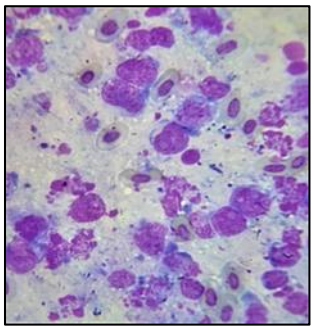

Fig 5: Impression smear-numerous pleomorphic lymphocytes and few lymphocytes with prominent nucleoli (H&E x1000)

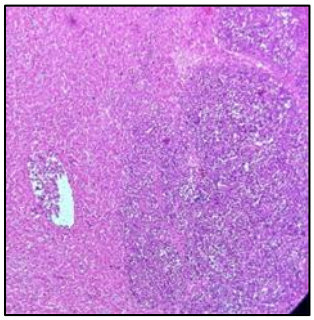

Fig 6: Liver - Pleomorphic lymphoid aggregates along with degenerative and necrotic changes of hepatocytes (H&E x100)

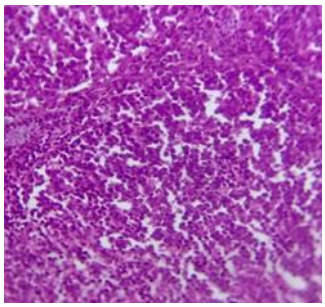

Fig 7: Spleen - Neoplastic lymphoid aggregates in germinal centres (H&E x400)

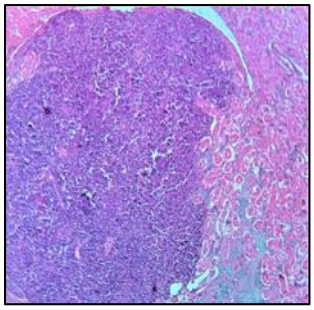

Fig 8: kidney - lymphoid aggregate displacing the renal parenchyma (H&E x100)

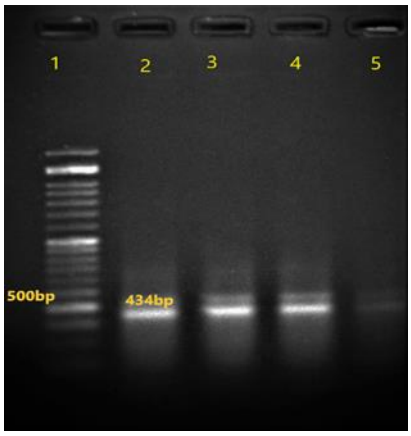

Fig 9: Agarose gel electrophoresis
Lane 1- molecular weight marker 1kb
Lane 2, 3, 4- PCR products from liver, spleen and kidney respectively
Lane 5- Negative control.

Conclusion

In the present study, MD was diagnosed pathologically and molecularly by PCR in unvaccinated chicken of the age group of 13–15 weeks. Hence, vaccination of desi birds is necessary but not sufficient for prevention of MD. A successful MD preventive programme in poultry, particularly desi birds, requires strict biosecurity practises to avoid exposure during the early stages of life.

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