STUDIES ON RETICULOENDOTHELIOSIS AND MAREK'S DISEASE IN POULTRY

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PATHOLOGY
(Minor Subject: Veterinary Microbiology)

By

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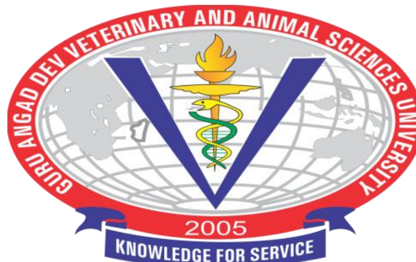
(L-2014-V-48-M)



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CERTIFICATE – I

This is to certify that the thesis entitled, **"Studies on Reticuloendotheliosis and Marek's Disease in Poultry"** submitted for the degree of **M.V.Sc.** in the subject of **Veterinary Pathology** (Minor Subject: Veterinary Microbiology) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Deepika Sharma (L-2014-V-48-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

The study conducted on 1619 birds' revealed tumours in visceral organs of 103 birds on necropsy with a prevalence of 6.36 %. Grossly, liver, spleen, kidneys, ovary, lungs, proventriculus and intestine showed either diffuse enlargement, enlargement with embedded foci or enlargement with varying sized greyish-white foci/ nodules. Sciatic nerves were enlarged in two cases, and no gross lesions were detected in brain. Microscopically, focal to diffuse infiltration of pleomorphic cells was observed in liver, spleen, kidneys, ovary heart, lungs, proventriculus and intestines. Brain showed gliosis, satellitosis, neuronophagia and perivascular cuffing, and nerves showed edema and focal aggregation of cells in one case. Immnunohistochemical staining using specific antisera against MDV and REV revealed positive reactivity for MDV and REV antigens in the cytoplasm and nucleus of the infiltrating cells in different visceral organs. 29/34 cases (85.3%) were positive for MD, 28/34 (82.3%) for RE, and 28/34 cases (82.3%) were positive for both MD and RE. Conventional PCR detected 29/34 cases (85.3%) of MD, 15 cases (44.12%) of REV, and 15 cases (44.12%) of both MD and REV. By multiplex PCR, 24 cases (70.59%) were positive for MDV, 15 (44.12%) for REV, 15 (44.12%) for both MDV and REV, and two cases (5.88%) for MDV, REV and ALV also. However, no insertion of REV genome into the MDV genome was detected by hot spot combined PCR. It was concluded that immunohistochemistry and PCR are required for differential diagnosis of mixed avian oncogenic virus infections under field conditions.

Keywords: ALV, avian neoplasms, immunohistochemistry, MDV, PCR, REV

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Signature of Major Advisor **Signature of the student**

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CHAPTER I

INTRODUCTION

Poultry production in India has taken a quantum leap in the last few decades, emerging from an unscientific farming practice to commercial production system. As per 19th Livestock Census, the total poultry population in India is 729.21 million and has increased by 12.39 per cent during the last decade. For the year 2014-15, egg production has been estimated to be around 78.48 billion and poultry meat production about 3.05 million tons (Annual Report 2015-2016). For the calendar year 2016, the egg production is forecast at 80 billion eggs and chicken meat consumption at 4.19 million tons. India's per capita consumption of poultry meat is estimated at around 3.1 kg per year and consumption of eggs is estimated at about 63 eggs per year (Poultry and Poultry Products Annual 2015). India is the ninth-largest producer of poultry meat, fifth largest egg producer and the eighteenth largest producer of broilers in the world. The demand for poultry in India is growing at 7 to 8 percent. The poultry sector has been growing at around 8-10 per cent annually over the last decade with broiler meat volumes growing at more than 10 per cent, while table egg is growing at 5-6 per cent driven by increased domestic consumption (http://foodprocessingindia.co.in/meat-and-poultry.html).

With the intensification of poultry industry the birds are exposed to production stress and rendered susceptible to various infectious and non-infectious diseases including neoplasms. Neoplasms are one of the most important causes of mortality and even lead to condemnation of carcass, resulting in huge economic loss to the poultry farmers. In poultry, neoplasms are caused mainly by avian oncogenic viruses that include viruses causing Marek's disease (MD), Reticuloendotheliosis (RE), lymphoid leukosis (LL) and lymphoproliferative disease (LPD) of turkeys (Davidson and Borenshtain 1999).

Reticuloendotheliosis is an oncogenic immunosuppressive disease of multiple avian species caused by reticuloendotheliosis virus (REV) belonging to family *Retroviridae*. These retroviruses are antigenically distinct from avian leucosis sarcoma group. REV belongs to gamma retrovirus and causes "runting" an acute non-

neoplastic syndrome in young birds characterized by high mortality, and severe immunosuppression or chronic neoplasia in adult birds with T and /or B cell lymphomas (Wang *et al* 2009). REV induced immunosuppression is of concern in commercial poultry production. Two types of tumours are commonly found in REV infected chickens; bursal and non- bursal. Bursal lymphomas are similar to those induced by avian leukosis viruses (ALVs), whereas the non–bursal lymphomas resemble to those induced by Marek’s disease virus (Witter *et al* 1986). The bursal tumours typically originate in the cloacal bursa, and later spread to liver and other organs within the period of 17-43 weeks. Non-bursal lymphoma occurs in visceral organs in chickens at about six weeks of age (Wang *et al* 2009).

REV is widespread and may be transmitted vertically, horizontally by direct contact with infected birds or mechanically by insects (McDougall *et al* 1981, Davidson and Braverman 2005). The viral RNA is first reversed transcribed in the cytoplasm (Asante-Appiah and Skalka 1997) and then the DNA intermediate inserts into the genomic dsDNA through the long terminal repeat (LTR) sequences which flank the retroviral genome (Coffin 1996). The LTR is the most stably integrated retroviral genomic fragment, as other parts are often excluded (Kost *et al* 1993). REV can integrate into the genome of large DNA viruses including the viruses that cause fowl pox and Marek’s diseases both of these diseases are controlled by vaccination in commercial poultry flocks (Witter and Fadly 2003). The vaccine strains carrying a nearly intact REV provirus are more likely to cause diseases in the field (Yuasa *et al* 1976, Jackson *et al* 1977, Isfort *et al* 1992, Witter and Fadly 2003).

Marek's disease, is a lymphoproliferative disease of birds characterized by multiple T-cell lymphoma formation in viscera, muscle, skin and lesions in peripheral nerves. It is caused by an oncogenic chicken alphaherpesvirus (Witter and Schat 2003). The classical form of the disease is characterized mainly by the involvement of peripheral nerves, whereas, the acute form is characterized by lymphoma formation in the visceral organs. Marek’s diseases virus (MDV) is ubiquitous and widely disseminated in poultry flocks worldwide. It is dsDNA avian herpesvirus and is one of the most economically significant pathogens of poultry with an estimated annual loss of up to US $2 billion worldwide (Davidson and Silva 2008). MDV replicates in the

nucleus and is present either as an open-form mini chromosome in cells supporting productive replication (Isfort *et al* 1992), as chromosome-integrated in transformed tumour cells (Delecluse *et al* 1993), or as both forms together (Rhiza and Bauer 1982).

Marek's disease virus (MDV) has been increasing in virulence since the mid twentieth century. In commercial poultry farming, MD is usually prevented by vaccination, which inhibits the onset of tumour formation, but not the virus infection. Vaccination dramatically reduced losses, but the disease remains one of significant pathogen, particularly because of the periodic appearance of new strains of MDV against which existing vaccines provide suboptimal protection. The mechanism of tumour prevention by vaccination still remains unclear (Morimura *et al* 1998, Baaten *et al* 2004, Davison and Kaiser 2004). If the protective immunity against MD was not established after vaccination, chickens would develop MD at younger ages, and consequently die. Since multiple vaccines have been developed and widely implemented, losses due to MDV have decreased. However, vaccine failure has occurred in the past and vaccine breakthroughs remain a problem. Despite of vaccination, failure of disease control with current vaccines results in significant economic and welfare consequences (Atkins *et al* 2013).

Lymphoid leukosis is a neoplastic viral infection of chickens caused by certain members of the leukosis/sarcoma group of avian retroviruses. These viruses are commonly called avian leukosis viruses (ALV) and belong to subgroups A, B, C, D, E, and J causing neoplastic diseases and other production problems in chickens (Fadly and Payne 2003). ALV (A-E) transforms B-lymphocytes, causing bursal lymphomas in chickens. ALV-J transforms myeloid cells causing myelocytomatosis, predominantly in bones mainly of meat type broilers.

Lesions caused by avian oncogenic viruses are similar and under field conditions multiple infection with different oncogenic viruses have been frequently reported (Kaur 2010, Gopal *et al* 2012, Mitra *et al* 2013, Wang *et al* 2014). The gross lesions in all the three diseases are almost similar and it is difficult to pin point the etiology. Although on the basis of microscopic lesions lymphoid leukosis and Marek’s disease can be differentiated, but the lesions of reticuloendotheliosis can be

confusing as these resemble the lesions of both LL and M.D. Therefore under field conditions RE remains under diagnosed. For differential diagnosis of avian oncogenic virus infections especially in case of mixed oncogenic virus infection, advanced pathological (Kaur 2010) and biomolecular techniques like conventional PCR (Mitra *et al* 2013), multiplex PCR (Gopal *et al* 2012), hot-spot PCR (Borenshtein and Davidson 1999) are employed.

Keeping in view confounding gross and pathological lesions produced by the avian oncogenic viruses and difficulties in the differential diagnosis of the mixed infection of avian oncogenic viruses under the field conditions, the present study was envisaged with the following objectives:

1. To study the prevalence of reticuloendotheliosis in chickens using molecular and pathological techniques.
2. To study the interaction of reticuloendotheliosis with Marek's diseases in poultry.

CHAPTER II

REVIEW OF LITERATURE

Neoplasms of poultry comprise a variety of related and unrelated conditions with common neoplastic characteristics. In addition to causing economic losses from tumour mortality as well as poor performance, some of these neoplastic diseases serve as highly suitable models for studying various phenomenon of neoplasia (Calnek 1986).

Neoplastic diseases of poultry fall into two broad classes, namely: those with an infectious etiology and those which are non-infectious. Those of the former category are of the greater economic importance because the viruses that cause these diseases are widely prevalent in commercial flocks and the mesenchymal neoplasms that they cause affect relatively young birds. These infections and diseases can be enzootic and epizootic. The non-infectious neoplasms are generally sporadic and not of great economic significance.

There are three economically important virus-induced neoplastic diseases of poultry, namely Marek's disease (MD), caused by a herpesvirus, and lymphoid leukosis (LL) and reticuloendotheliosis (RE), caused by retroviruses (Fadly 2005). A neoplastic disease of turkeys known as lymphoproliferative disease (LPD) that had been reported in Europe and Israel and is induced by another retrovirus distinct from both the leukosis/sarcoma and reticuloendotheliosis viruses.

Neoplasms of unknown etiology are classified according to their morphologic characteristics; they include a wide variety of benign and malignant neoplasms such as dermal squamous cell carcinoma, multi-centric histiocytosis and adenocarcinoma (Fadly 2008).

2.1 Marek’s disease

Marek’s disease is the most common lymphoproliferative disease of chickens caused by herpesvirus (Biggs 1961) and transmitted horizontally via the respiratory route (Beasley *et al* 1970). The disease was first time reported in 1907 from United States and later reported from Netherlands, Great Britain and many other countries of

the world. It occurs in two forms i.e. acute and chronic. Young birds seem to be highly susceptible to acute form of disease (Lobago and Woldemeskel 2004).

A herpesvirus termed Marek's disease virus (MDV) was found to be associated with the tumours and shown to be the etiologic agent (Churchill and Biggs 1967, Solomon *et al* 1968, Calnek *et al* 1970). MDV infection in chickens leads to the formation of T cell tumours in various body tissues, neurological manifestations as well as immune suppression (Calnek 2001, Payne 2004).

MDV isolates were categorized into three classes based on serological characteristics; oncogenic strains (serotype-1), non-oncogenic MDVs from chickens (serotype-2) and the herpesvirus of turkeys (HVT) related non- oncogenic serotype-3 (Witter *et al* 1970a). Shortly after MD herpesviruses were isolated, some serotype 1 attenuated (Churchill *et al* 1969), and serotype 3 (Okazaki *et al* 1970) viruses were developed as successful cell-associated vaccines against MD, these were the first successful vaccines used worldwide to combat 8 tumours in any species (Calnek and Witter 1984). Later, when more virulent forms of MDV were described (Witter *et al* 1980), the serotype 2 viruses were also developed and used, mainly as bivalent vaccines (Witter 1982, Calnek *et al* 1983).

Feather dander and poultry house dust served as the source for inhalation of infectious MDV and subsequent establishment of natural infection (Beasley *et al* 1970, Calnek *et al* 1970). Initial respiratory infection was followed by the cytolytic phase that extends from 3-6 days post infection and occurs in lymphoid organs such as spleen, the bursa of Fabricius and thymus (Calnek 2001). MDV from respiratory system carried to other organs by infected macrophages (Barrow *et al* 2003) where initially B-cells were infected by MDV (Shek *et al* 1983, Calnek *et al* 1984, Baigent *et al* 1998). Following a burst of productive/restrictive infection in B cells, a switch to latent infection in T cells occur about 7 days post infection. The switching of infection may be influenced by a protein encoded by MDV, viral interleukin-8 that acts as a chemoattractant for chicken T cells (Liu *et al* 1999, Parcells *et al* 2001) and allows the infiltration of T cells to the vicinity of MDV infected B cells.

MDV infected cells can upregulate major histocompatibility complex class II molecules on MDV infected cells (Niikura *et al* 2007), which may facilitate

presentation of MDV antigen (Malnati *et al* 1992) and initiation of host response, allowing T cell infiltration into the site of virus replication. MDV infection in T cells becomes latent which is followed by transformation of T cells and tumour formation in Marek's disease susceptible chicken (Calnek 2001). These lymphocytes migrate through the bloodstream to visceral organs and peripheral nerves where they become neoplastic and, in susceptible chickens, proliferate to form gross lymphomas 3–4 weeks post-infection (Sevoin *et al* 1962, Payne and Biggs 1967). Ten days post-infection onwards, MDV undergoes fully productive replication in the feather follicle epithelium and high levels of MDV antigens are expressed, and cell-free virus shed with skin and feather debris throughout the life of an infected bird (Calnek *et al* 1970). The cytolytic phase is an important phase of the MDV replication cycle that mainly involves the bursa of Fabricius (Schat *et al* 1981) and the consequence of this phase is usually immuno-suppression leading to increased susceptibility to other secondary infections (Islam *et al* 2002).

Pathological lesions of Marek's disease

Benton and Cover (1957) studied acute form of MD in broiler chickens, and observed tumours in multiple viscera, muscle and skin.

Wight (1962) classified the nerve lesions into three types, two of which were essentially inflammatory or degenerative while the third one was neoplastic. Type I lesions were charecterised by cellular infiltration and relatively little edema. Most cells were small lymphocytes or plasma cells but there were some lymphoblasts in cases of massive infiltrations. In Type II, edema was marked and only a few infiltrating cells (mostly plasma cells) were present. Fibrosis was occasionally seen. In type III lesions there was massive infiltration of lymphoblastic cells and the observation of frequent mitoses. All three types were considered histologic variants of the same condition, but it was thought that the neoplastic changes followed the inflammatory lesions.

Payne and Biggs (1967) observed proliferative lymphomatous lesions in the visceral organs. The cellular composition consisted of diffusely proliferating small to medium lymphocytes, lymphoblasts, and primitive reticular cells in the field cases of MD.

Changes in the Bursa of Fabricius and thymus of experimentally infected birds were reported by Jakowski *et al* (1969). In the bursa there were cortical and medullary atrophy, necrosis, cyst formation and interfollicular lymphoid infiltration. Atrophy of the thymus was sometimes severe and also involved both the cortex and medulla. In some cases there were areas of lymphoid proliferation in the thymus

Fujimoto *et al*. (1971) observed tumorous nodules (pin-point to 2 mm in diameter), greyish-white in color on the liver, spleen, ovary and kidney parenchyma and were firm in consistency. These tumorous nodules were present in birds suffering from acute MD without involvement of peripheral nerves.

Sharma *et al* (1972) reported the skin form of MD in naturally as well as experimentally infected birds and described gross pathology and histopathology of skin lesions in four spontaneous cases of MD. Grossly, the feather follicles were enlarged and skin surface had varying sized tumorous growth.

Dutton *et al* (1973) observed that Marek's disease was characterized grossly by single to multiple, white to light yellow/greyish nodules in different visceral organs. Gross lesions of Marek's disease in Japanese quail were mainly localized in the liver, spleen, kidneys and small intestine and characterized by nodular or diffuse enlargement (Fujimoto *et al* 1975).

Sharma *et al* (1976) revealed that infiltrating pleomorphic lymphoid cells, plasma cells and histiocytes completely obliterating the normal architecture in different visceral organs viz. liver, spleen, proventriculus, lungs, intestine, gonads, kidneys and nerves

Classical form of MD characterized mainly by neural involvement i.e. peripheral nerves. The most commonly involved nerves include brachial and sciatic plexuses, coelic plexus, abdominal vagus and intercostal nerves (Sahota and Singh 1976).

Mladenov *et al* (1977) observed that lymphoid proliferates of MD were pleomorphic lymphocytic cells whereas, lymphoid proliferates of LL were lymphoblastic in various organs.

Rao and Sarma (1984) observed liver and spleen as the most affected organs in Marek's disease in layer flock characterized by single or multiple nodules in liver, spleen, kidneys, ovary, proventriculus, bursa, heart, nerves, skeletal muscles and eye.

Pradhan *et al* (1985) examined natural cases of Marek's disease in Japanese quail and reported that lesions were confined to the liver and spleen. Nair *et al* (1986) studied MD in Japanese quails and observed diffusely enlarged yellowish white liver with greyish white nodules. Spleen was greyish-white and many times enlarged to its normal size.

Kobayashi *et al* (1986) studied lymphomatous lesions characterized by diffuse infiltration and proliferation of lymphoid cells of different sizes and perivascular cuffing in brain. Neuhybel *et al* (1986) experimentally induced Marek's Disease and observed that lymphomas of MD were infiltrative and seen mostly in perivascular location with marked pleomorphism.

Moriguchi *et al* (1989) observed that in contrast to the visceral lymphomas, lesions of the skin were more inflammatory. Sometimes in addition to the massive accumulations of mononuclear cells around the feather follicles, complete aggregates of proliferating cells, often perivascular, and a few plasma cells and histiocytes were seen in the dermis. The massive proliferative lesions may cause disruption of the epidermis resulting in an ulcer.

In studies on transient paralysis, Swayne *et al* (1989) described a vasculitis leading to vasogenic edema. Lesions were most consistently seen in the cerebellum. Ultrastructural changes did not include demyelination

Imai *et al* (1990) studied 61 Japanese quail with problems of recurring outbreaks of lymphoproliferative diseases. They observed lymphomatous lesions in 17 quails and detected MDV antigen in feather tips and MD antibodies in quails. SPF chicks inoculated with blood of the infected birds developed MD and MDVs were detected by indirect immunofluorescent antibody test.

Panneerselvam *et al* (1990) observed Marek's disease in 496 (5.78%) of 8582 layers studied. The most commonly affected organs were the liver (82%) and spleen (74%). Other affected organs included the kidneys, heart, proventriculus, ovaries, lungs and nervous system. Mortality was higher among 9-20 weeks old birds.

Ficken *et al* (1991) and Spencer *et al* (1992) reported MD outbreaks in which blindness was a common feature with extensive infiltration by inflammatory cells in

the cornea, uveal tract, retina and pectin, intra-nuclear inclusion bodies in mononuclear cells and retinal cells and degeneration of photoreceptors and other retinal cells.

Cho *et al* (1999) described histopathologic changes in the brain. Lesions were always focal in distribution and consisted of either compact perivascular cuffs of small densely staining lymphocytes or submilliary nodules composed of lymphocytes and pallor element.

Narang *et al* (2003) studied MD in layer flocks and observed small to large sized greyish-white tumours on liver, spleen, kidneys, heart, lungs and thickened sciatic nerves.

Gurel *et al* (2003) observed lymphoid cell infiltration of various sizes particularly in liver and to lesser extent in kidneys, spleen, proventriculus and sciatic nerves

Goyal (2004) observed Marek's disease in adult layers and quails and among all lymphoproliferative diseases, acute form of Marek's disease was dominated, and was characterized by both progressive and regressive lesions in different tissue, besides vascular invasion and embolization by tumour cells.

Lesions in peripheral nerves consisted of light to heavy mononuclear cells, sometimes associated with edema, myelin degeneration and Schawann cell proliferation. Axonal degeneration was rare. The offending cells were usually a mixture of several types including, small and medium lymphocyte, plasma cells, and lymphoblast (OIE, 2004)

Pejovic *et al* (2007) described the macroscopic characteristics of Marek's disease (MD) tumours in 20 chickens aged 8 to 24 weeks. The affected organs were enlarged, compact and very brittle, irregular in shape, greyish-red or greyish- white and of fatty consistency. Most often characteristic changes were in the spleen, liver, proventriculus and ovary that usually had a compact or ribbed, fatty cauliflower like formation. In histopathology there was proliferation of small and medium size lymphocytes, lymphoblast cells, MD cells and activated reticular cells.

Shahzad *et al* (2007) observed neoplastic lesions most commonly in peripheral nerves, iris, gonads, spleen, heart, lungs, liver and muscle. Diagnosis was based on enlarged nerves, lymphoid tumours in viscera and confirmation was by demonstration of tumour-associated surface antigen on some of the individual cells by immunofluorescence.

Balachandran *et al* (2009) conducted histopathological examination of 767 tissue samples collected from different parts of India and Bangladesh. Out of 767 tissues, 73 tissues were from broiler chickens and 694 from layer chickens. Prevalance of Marek's disease lesions was found to be more in the liver (34.34%), followed by spleen (26.26%), kidneys (12.12%), proventriculus (8.08%), ovaries (7.07%), lung (4.04%), sciatic nerve (3.03%), intestine (2.02%), skin (1.01%) and mesentery (1.01%).

Hablolvarid (2011) examined tissues and organs of slaughtered chickens from a flock of 80 birds, both grossly and histopathologically and found 24 (80 %) birds positive for MD in different forms. Incidence of cutaneous, visceral and mixed cutaneous and visceral forms of the diseases was noted as 16.2%, 3.8% and 10%, respectively while no case of nervous and ocular forms was seen.

Mahabubulalam (2012) reported large miliary whitish nodular lesions in liver, spleen, lungs and intestine. Enlargement of sciatic nerve and thickening of feather follicle was commonly observed.

Singh *et al* (2012) reported that Marek's diseases was highly contagious and neuropathic disease of chickens resulting in development of tumours in visceral organs characterized by development of CD4+T cell lymphomas as well as infiltration of nerves and visceral organs by lymphocytes. Pleomorphic cell infiltration in peripheral nerves, gonads, lymphoid organs, iris, muscle, skin and other visceral organs was observed.

Musa *et al* (2013) conducted retrospective study on Marek's diseases and found its occurrence during pre-rainy to rainy season. The clinical analysis revealed uneven growth, progressive weight loss, ruffled feathers and whitish yellow diarrhea. Major gross lesions were severe emaciation, thickened proventriculus and flabby

heart with loss of coronary fats. MD was mainly observed in chickens aged from10 to 20 weeks.

Abreu *et al* (2016) detected Marek's Disease in a free range poultry based on macroscopic and microscopic lesions. Tumour lesions were observed in liver, ovary, kidney, spleen and skin. In breast muscles, whitish lesions in streak form were observed. Hughes *et al (*2016) studied six-month-old female turkey from a commercial growing unit for investigation of suspected viral lymphoproliferative disease and observed diffusely enlarged spleen, kidneys and liver, with multifocal creamish foci. The lungs were atelectatic, and mild segmental thickening of the small intestines was observed. The case was diagnosed as Marek's disease.

2.2 Reticuloendotheliosis

Reticuloendotheliosis designates a group of pathologic syndromes in several avian species caused by reticuloendotheliosis virus (REV), a member of the avian retrovirus group. The initial REV isolate, strain T, was obtained in 1958 from a turkey with visceral lymphomas, and was serially passaged over 300 times in turkeys and chickens (Robinson and Twiehaus 1974). The respective disease was named as "reticuloendotheliosis" on the basis of prominent cell in the neoplastic lesion (Theilen *et al* 1966).

REV is an avian retrovirus with multipotent pathogenic potential. The first syndrome is an acute reticulum cell neoplasia induced in chickens and other avian species only by strain T (Robinson and Twiehaus 1974), a replication-defective variant containing the viral oncogene, *v-rel* (Hoelzer *et al* 1979). The second syndrome, a runting disease (Witter *et al* 1970b) caused by nondefective RE virus, is characterised by one or more non-neoplastic lesions including runting, bursal and thymic atrophy, enlarged peripheral nerves, abnormal feather development, and general immunosuppression. A third syndrome is the chronic B-cell neoplasia that is induced in chickens by non-defective RE viral strains and closely resembles lymphoid leukosis (Witter and Crittenden 1979, Witter *et al* 1981). The chronic neoplastic lesions are characterized by involvement of the bursa of Fabricius, have relatively long latent periods, are of B-cell origin as determined by the presence of surface IgM (Witter *et al* 1981) and susceptibility to ablation by chemical or surgical bursectomy

(Fadly and Witter 1983), and are usually associated with activation of the *c-myc* gene caused by adjacent insertion of proviral DNA in the cellular genome (Noori-Daloii *et al* 1981, Swift *et al* 1985).

REV due to its immunosuppressive effects may predispose affected birds to a variety of secondary infectious agents (Witter and Fadly 2003). REV can integrate into the genome of large DNA viruses including the viruses that cause fowlpox and Marek's disease both of these diseases are controlled by vaccination in commercial poultry flocks (Witter and Fadly 2003). The vaccine strains carrying a nearly intact REV provirus are more likely to cause disease in the field (Singh *et al* 2003, Witter and Fadly 2003). The clinical outcome of REV infection ranges from a runting syndrome to acute reticulum cell neoplasia or to generation of T-cell or B-cell lymphomas that may resemble those induced by Marek's herpesvirus or avian leukosis/sarcoma viruses (Witter *et al* 1986, Witter and Fadly 2003).

Pathological lesions of Reticuloendotheliosis

Grimes and Purchase (1973) observed enlarged and mottled liver and spleen with multiple yellowish white foci and tumorous lesions in duodenum, pancreas and wall of proventriculus in duck infected with reticuloendotheliosis. Microscopic observation was mononuclear cell infiltration in liver and spleen predominantly mononuclear lymphoreticular cells

Robinson and Tweihaus (1974) detected enlarged liver and spleen with white foci in REV infected birds. On histopathological examination pleomorphic primitive cells in the interstitial tissues to form small nest or nodules. REV infection was charcterized by the infiltration and proliferation of large vesicular cells, variously described as mononuclear cells of the reticuloendothelial system

Carlson *et al* (1974) detected reticuloendotheliosis in a flock of Japanese quail. Most striking and consistent lesion was marked thickening and nodule formations along the digestive tract in addition to this, tumour masses in the lungs, liver, spleen, heart, pancreas, caecal tonsils, ovary, kidney, airsacs, thyroid glands, testes and sciatic nerves was also observed. Microscopic examination revealed proliferation of anaplastic mononuclear type cell in the affected tissues. The cells bore all the

characteristics of neoplastic growth, with large bizarre nuclei, numerous mitotic figures, misshapen nucleoli, and prominent pleomorphic cytoplasm.

Paul *et al* (1976) studied reticuloendotheliosis in a turkey flock. In naturally affected turkeys, lesions were present in the liver, spleen, heart, intestines, and peripheral nerves, and were composed mainly of lymphoreticular cells

Tajima *et al* (1976) observed induction of abnormal feathers, characterized by thinness and increased transparency of the calamus and rachis, and loss of barbs, at a high frequency by inoculating day old chicks with REV propagated in chicken embryo fibroblast cultures. Lesions of an inflammatory degenerative type were observed in close association with the presence of viral antigen and numerous C type virus particles, characteristics of REV, in the intermediate and cylindrical cell layers of all abnormal feathers examined.

Solomon and Nazerian (1976) detected REV from turkey lymphoid tumours. The REV isolates were culturally and antigenically similar to the prototype REV strain. Data supported an etiological role for REV in some though not all forms of turkey neoplastic disease.

McDougall *et al* (1978) studied leukosis and REV from turkeys. Clinically diseases was characterised by enlargement of the liver and microscopically the lesions consisted of proliferations of lymphoblastoid cells. REV was isolated from ailing culled turkeys and from cell cultures prepared from embryonated eggs produced by a flock with this disease. REV was isolated from affected flock in which enteritis was the main clinical feature. Inoculation of 1-day-old turkeys with this isolate of REV produced a syndrome of enteritis followed by leucosis.

Ratnamohan *et al* (1980) found multiple cream coloured nodules in intestine, liver and spleen of a reticuloendotheliosis infected hen.

Witter and Glass (1984) studied REV from a flock of breeder turkeys at 21 weeks with lymphoid neoplasms. Egg transmission seemed unlikely, because no REV antibodies were observed in flocks (hatchmates) and in three progeny flocks. Eggs obtained from the breeder flock at 45 weeks lacked REV group-specific antigen and produced poults negative for REV infection. The virus isolate, designated REV strain 339, produced an acute neoplastic disease when inoculated into young chickens.

Okoye *et al* (1993) observed outbreaks of clinical reticuloendotheliosis (RE) in a turkey flock and two pullet flocks. Clinical signs in turkeys included sleepiness, weakness, anorexia, diarrhoea and reduced egg production. The pullets showed severe emaciation, loss of back feathers, anorexia and diarrhoea. On necropsy examination dead turkeys and pullets showed neoplastic nodules or grey foci in the internal organs especially the liver, intestines and spleen. Histopathological sections of the organs revealed proliferation of reticular cells with necrosis of the parenchyma and focal lymphocytic infiltration. REV antigens and precipitating antibodies were detected in the serum samples collected from the birds

Zavala *et al (*2006) detected RE in the endangered Attwater's and Greater Prairie chickens. The observation was prairie chickens affected with a neoplastic condition. Investigative procedures included postmortem examinations, histopathology, molecular detection, and virus isolation. REV infection was diagnosed using virus isolation and PCR. Lymphosarcomas observed on histopathology. REV was confirmed as a significant cause of mortality in captive prairie chickens

Lin *et al* (2009) studied REV in a white Roman goose farm in Taiwan. Observation included nodular lymphoma-like tumours in the liver, lung, kidney, and pancreas. Further confirmation was through PCR.

Wang *et al (*2012) experimentally inoculated day-old specific pathogen-free (SPF) White Leghorn chickens with REV-JX0927 to evaluate pathogenicity and oncogenicity of the virus using hematology, serology, histopathology immunohistochemistry and apoptosis assay. Histopathology suggested that REV-JX0927 induced reticuloendotheliosis at early stage (1week), and lymphosarcomas at middle stage (after 7 weeks). In addition, squamous-cell carcinoma, adenocarcinoma and aneurysm were found in infected birds. It was noted that REV-JX0927 caused new pathogenitic characters in infected birds it was concluded that REV was a multipotential oncogenic retrovirus.

Gopal *et al* (2015) studied reticuloendotheliosis in natural cases of tumours in body. They collected Liver, spleen from suspected chicken and turkey farm around

Namakkal, Tamil Nadu. Histopathological examination revealed homogenous monomorphic lymphoid infiltration which was further confirmed by IHC and PCR.

Moshira *et al* (2016) studied REV in chicken flocks reared in two provinces in delta Egypt. About 691 birds were submitted for pathological investigations. Total of 691 birds 278 birds (40.2%) had unusual tumour formation in the head, neck regions and enlarged liver, spleen, kidneys. Visceral lymphomas occurred in a 25 weeks old chicken flock. The common pathologic lesions were multiple neoplastic nodules in the livers and spleen consisting of homogeneous lymphocytes. Histopathology of the head region revealed hypercellularity with intercellular transudate, focal aggregation of inflammatory cells around the diluted blood vessels and haemorrhages in tumour mass. The total WBCs, heterophils and monocytes count were significant higher in birds with tumours.

2.3 Lymphoid leukosis

Avian lymphoid leukosis is a neoplastic disease of chickens caused by a virus of the leukosis/sarcoma group and characterized by tumour formation of the bursa of Fabricius with metastasis to other tissues and all abdominal organs. Avian leukosis virus (ALV) is the most common naturally occurring avian retrovirus, causing neoplastic diseases and other production problems in chickens (Fadly and Nair 2008). ALVs are the member of the retroviridae family (Goff 2001). Based upon differences in their envelopes, ALVs are divided into six subgroups. Subgroups A, B, C, D and J are classified as exogenous viruses and can induce B-cell lymphomas in susceptible chickens. Subgroup A viruses are the most common subgroup found in field flocks (Fadly and Payne 2003). In 1989, a subgroup J ALV was isolated in the U.K. (Payne *et al* 1991). Subsequently ALV-J spreads throughout the world, resulting in severe economic losses in the commercial breeder industry. Subgroup E viruses are endogenous ALVs present in nearly all chicken genomes and induce little or no oncogenecity. Exogenous avian leukosis viruses are type C retroviruses associated with a variety of neoplasms including lymphoid and myeloid leukosis. Unlike endogenous viral genes that are inherited as host genes, spreading of exogenous ALVs in commercial poultry is through vertical and horizontal transmission (Crittenden 1981).

Subgroups A and B are the most common ALVs in commercial poultry (Calnek 1968). Subgroups C and D have been rarely reported in commercial poultry (Payne and Fadly 1997). Subgroup E viruses include the ubiquitous endogenous leukosis viruses of low pathogenecity (Smith 1987). ALV-J behaves as an exogenous virus causing mainly myeloid leukosis and nephromas in meat type chickens (Payne 1992, Fadly and Smith 1999). Exogenous ALVs are capable of inducing a variety of neoplasms; under natural conditions, lymphoid leukosis (LL), a B-cell lymphoma that originates in the bursa of Fabricius and metastasizes to viscera is the most common neoplasm induced by this group of viruses (Ewert and deBoer 1988, Fadly and Nair 2008). The pathogenesis of ALV induced lymphoid leukosis involves the formation of pre-neoplastic bursal follicles, eventually these pre-neoplastic bursal follicles develop into bursal lymphoma and metastize to various organs (Fadly and Payne 2003). Chickens infected with exogenous ALV shed virus into albumen of eggs, vaginal and cloacal secretions, and congenitally transmit virus to the next generation. In contrast, endogenous ALVs are believed to be not shed or shed at a very low level. ALV-J is believed to have arisen by recombination between exogenous ALVs and the endogenous retroviruses (Bai *et al* 1995a, Bai *et al* 1995b). Sequence analysis of more recent isolates of ALV-J has revealed multiple changes in the *env* gene leading to antigenic variation (Venugopal *et al* 1998).

Grossly, in lymphoid leukosis, liver appeared fragile, enlarged, with greyish white milliary foci of varying size on the surface as well as in parenchyma. Kidneys also showed nodular growths and urate deposits in parenchyma (Ghosh *et al* 1985) Spleen and kidney appeared enlarged and mottled. Ovaries revealed enlargement, haemorrhages and nodular growths (Lakhotia and Mehta 1988). Liver showed several smooth soft nodular growths in few cases, bursa was also enlarged and sometimes with raised discrete nodules (Rai *et al* 1990). The gross pathological lesions of ALV-J characterized by skeletal myelocytomas located on the inner sternum and ribs, neoplastic enlargement of liver, and in some cases gross tumour involvement of the spleen, kidney, trachea, skeletal muscles, bone marrow, skin and gonads (Bagust *et al* 2004).

Histologically, lymphomas of LL are characterized by uniform sized lymphoblasts in different visceral organs viz. heart, lungs (Mladenov *et al* 1977) liver, spleen (Ghosh *et al* 1985), kidneys, ovary, intestine and bursa (Markan 2003) along with degenerative changes. (Rai *et al* 1990) observed infiltration of lymphoid cells especially lymphoblasts in liver, spleen, kidneys and other visceral organs with no change in sciatic nerves The tumours of ALV-J consisted of immature granulated myelocytes, and showed focal or diffuse infiltrations in the affected organs (Bagust *et al* 2004).

2.4 Mixed infection of avian oncogenic viruses.

Isfort *et al* (1992) observed retrovirus can integrate directly into a herpesvirus genome. Specifically, it was demonstrated insertion of a non-acute retrovirus, REV, into a herpesvirus, Marek's diseases virus (MDV).

Jones *et al* (1993) studied that REV can integrate into the MDV genome following both long and short term co-infections of chicken or duck embryo fibroblasts (DEF). It was observed that herpesvirus are large double – stranded DNA viruses that can carry foreign sequences. Integration of retroviral sequences into MDV occurs mostly at the junctions of the unique and repeat sequences known as hotspots of integration.

Isfort *et al* (1994) indicated that retroviruses can integrate into and mutate the genomes of herpesvirus during co-infection. This interaction has the potential to change the host range and pathogenicity of both viruses and result in novel infectious agents and diseases. This phenomenon also allows genetic material to be exchanged between these viruses and their host.

Aly *et al* (1996) reported that the incidence of REV induced bursal lymphomas was significantly higher in chickens vaccinated with serotype-2 MDV than in unvaccinated chickens or chickens vaccinated with HVT.

The survey of commercial tumour bearing birds for avian oncogenic viruses indicated that both herpesviruses and retroviruses could infect the same bird under natural conditions (Davidson *et al* 1998)

Davidson and Borenstein (1999) conducted a 5-year retrospective study that included 207 chicken and 52 turkey flocks. They detected MDV and several retroviruses including REV, ALV in chickens and lymphoproliferative disease virus

in turkeys and observed these were oncogenic and immunosuppressive agents. These viruses were detected either alone, or in various combinations in blood and tumour DNAs of commercial birds by PCR.

Cui *et al* (2006) demonstrated the spontaneous occurrence of MDV and retrovirus chimeras in Chinese commercial flocks that had spread to commercial chicken flocks. The recombinant viruses were re-isolated in tissue culture and shown to contain retroviral LTR inserts of 540bp. These chimeras apparently caused a more severe thymus and bursa of Fabricius atrophy than was expected.

Singh *et al* (2010) conducted a study on field avian oncogenic virus infections using polymerase chain reaction and immunohistochemistry. In majority of the cases, a mixed infection of MDV with oncogenic avian retroviruses was observed under field conditions. They further reported that most of the outbreaks of MD occurred in adult laying birds (>20 weeks of age). By using immunohistochemistry and multiplex PCR. Gopal *et al* (2012) detected multiple infections of avian oncogenic viruses under field conditions

Mitra *et al* (2013) detected the presence of multiple oncogenic virus infection from blood of 25 apparently healthy chickens with PCR. Concurrent presence of DNA of multiple oncogenic viruses included MDV, REV, avian leukosis virus was found in the same bird and it was concluded that PCR can be utilized to detect latent infection of avian oncogenic viruses in birds.

Buscaglia (2013) observed the presence of REV in flocks affected with Marek's disease (MD). Sera were positive to REV antibodies by agar gel precipitation. Frozen sections from tumours were positive for MDV but were negative for REV. Chicken embryo fibroblast (CEF) and chicken kidney cell (CKC) culture inoculated with buffy coat cells or blood from the affected birds were examined. Positive cells were shown for REV and MDV by fluorescent antibodies tests in CEF and CKC, respectively, indicating the presence of REV in Argentinean layer flocks.

Bao *et al* (2015) obseved mixed infections of REV and MDV in China and reported that found REV causes a series of pathological syndromes, especially the immunosuppression of the host, which may lead to an increased susceptibility to other pathogens..

2.5 Diagnostic techniques for detection of avian oncogenic viruses.

I. Immunohistochemistry

Cho *et al* (1999) inoculated specific pathogenic free birds (SPF) at 1 day of age and observed development of skin lymphoma and demonstrated Marek’s disease virus particles with in necrotic lymphoblasts with the help of electron microscopy and immunohistochemistry.

Pejovic *et al* (2007) described immunophenotypic characteristics of Marek’s disease (MD) tumours in chickens aged between 8 to 24 weeks of age. Immunohistochemical analysis revealed that lymphoma cells in MD were of CD3+ phenotype.

Santos *et al* (2008) used immunohistochemistry and in situ hybridization for detection of reticuloendotheliosis virus infection in experimentally inoculated Japanese quails embryo. It was reported that both these assays were efficient for diagnosis of active reticuloendotheliosis virus infection, but were found to be less sensitive when applied to archieved tissue samples.

Xiu-guo *et al* (2008) inoculated MDV and REV in broiler breeders and studied pathological changes, apoptosis, immunohistochemistry (immune-fluorescence), and ultrastructure of tumour tissues of the affected birds. It was concluded that differential diagnosis of these two diseases could be done by immunohistochemistry at an early stage (before 2 weeks), and histopathology in the later stage (post 4 weeks). They noticed proliferation of lymphocytes at age of 1 week and Marek’s disease cells (MDCs) were observed at 2-9 weeks.

Wang *et al* (2012) used immunohistochemistry in experimentaly REV-JX0927 inoculated day-old specific pathogen-free (SPF) White Leghorn chickens to evaluate pathogenicity and oncogenicity of REV. It was concluded that REV-JX0927 was a multipotential oncogenic retrovirus.

II. Polymerase chain reaction

Oncogenic and attenuated variants of serotype 1 Marek's disease viruses MDV can be differentiated by the polymerase chain reaction amplification of the 132bp tandem direct repeats within the MDV genome (Silva 1992).

A polymerase chain reaction (PCR) test using primers flanking 132 bp tandem repeats in pathogenic MDV DNA was developed by Becker *et al* (1992). These primers amplified a dimmer or a trimer 132 bp repeats in pathogenic MDV-1 DNA from blood and organs of commercial chicken with symptom of MD. Using the same primers in a radioactive PCR test it was possible to distinguish between very virulent MDV -1 and the non- pathogenic MDV-1 (CVI-998vaccine virus) in which, the 132 bp repeats were found more, i.e up to nine repeats. The MDV-1 specific primers did not amplify MDV -2 (SB-1) and MDV-3 (HVT) DNA. It was concluded that PCR could be used for early identification of vvMDV-1 in pathological samples from diseased commercial chicken and to distinguish between the vvMDV-1 and three types of virus vaccines used to immunize chicken.

Zhu *et al* (1992) used PCR for the differentiation of oncogenic and non-oncogenic strains of MDV type 1, using primers chosen from the sequences within the long inverted repeats of MDV-1 DNA. Oncogenic strain infected cells and MD tumours cell lines produced a major product containing two or three copies of 132 bp repeats, whereas the non oncogenic strain infected cells yielded amplified products with various sizes corresponding to the number of 132 bp repeats. The primers chosen from the glycoprotein A genes of MDV 1 and HVT were also used for determination of their serotype specificity. The PCR procedure was found to be simple and sensitive for identification of MDV-1 and HVT, as well as for estimation of oncogenicity of MDV-1.

Fu *et al* (1993) studied PCR assay specific for Marek's Disease Virus which was able to detect MDV in inoculated chick kidney cells at dilutions of 10^5. Bursa, feathers and kidneys from MDV infected chickens were positive in the PCR assay. Internal probe hybridization also confirmed that the PCR products were from MDV.

Aly *et al* (1993) used PCR for detection of REV. DNA extracted from chick embryo fibroblasts (CEFs), blood and tumours of chickens experimentally infected with the spleen necrosis virus (SNV) strain of REV was used as the target for chain elongation. They concluded that PCR was a sensitive and specific method for detection of REV infection and tumours.

Davidson *et al* (1995) studied normal and tumour-bearing birds from a total of 16 chicken and turkey flocks using PCR for differentiating between MDV and REV. All chickens were examined for REV antibodies by ELISA. REV isolation was performed in CEF and the tumours were examined histopathologically. MDV and REV- PCRs were performed on whole blood DNA and tumour DNA from the same bird. It was observed PCR was effective and sensitive method for differential diagnosis of tumour-bearing and immuno-deficient birds.

Takagi *et al* (1996) applied the reverse transcriptase polymerase chain reaction (RT-PCR) to detect contamination of MD vaccine with REV and found the sensitivity of the RT-PCR system to be higher than that of the flourescent antibody assay.

Bumstead *et al* (1997) developed quantitative assay for Marek's disease virus which determined the number of viral genome in a sample by PCR. Fluorescent tagged primers for PCR amplification were used. This technique was found to be rapid, less laborious with high level of accuracy as compared to plaque assay.

Davidson *et al* (1998) reported the advantage of PCR application for differential diagnosis of avian oncogenic viruses. The survey of commercial tumour bearing birds for avian oncogenic viruses indicated that both herpesviruses and retroviruses could infect the same bird under natural conditions. PCR detected avian oncogenic viruses either alone, or in various combinations in blood and tumour DNAs of commercial birds.

Reddy *et al* (2000) developed a quantitative competitive PCR for the detection of MDV. The assay utilized a competitor DNA that differed from the viral DNA of interest by having a small insertion, which acted as internal standard for the estimation of viral DNA in an unknown sample. It was found that a more virulent strain of MDV (648A) replicated better in thymus during cytolytic infection then did a less virulent strain.

Kozdrun *et al* (2001) used three different reactions and primers for gene A in PCR to differentiate MDV strains belonging to serotypes 1 and 3, and also the distinction of virulent and attenuated strains within the limits of serotype 1.

Kalyani (2006) differentiated MDV serotype-1 from four MDV vaccine strains i.e. three HVT (MDV-3) vaccine strains and one SB-1 (MDV-2) vaccine strain by PCR and also compared the efficacy of different primers in detecting MDV.

Fadly *et al* (2008a) applied PCR using primer sets to amplify a 2135 base pair fragment overlapping the REV envelope region and partial long terminal repeat region and noted a high degree of DNA sequence homology between and among the three REV isolates from broiler breeder chickens, turkeys and prairie chickens located in Alabama, California and Texas, respectively. Greater than 99.0% homology at the nucleotide level was observed between the three isolates and strain CSV (chicken syncytial virus), a prototype of REV subtype C, thus suggesting that these REV isolates although obtained from different avian species located at three different locations in the USA were closely related genetically

Raja *et al* (2009) collected a total of 173 samples of feather follicles, spleen, and liver from poultry in three states and studied virulence of MDV strains in the Indian poultry flocks. 22 liver samples were positive for MDV strain 1 in PCR and *in situ* hybridization, respectively. Moreover, flow cytometry results showed that virulent MDV induced apoptosis more efficiently than HVT vaccine virus.

Mays *et al* (2010) studied nine REV isolates from broiler breeders, turkeys, and prairie chickens from USA, and three isolates obtained from known contaminated live-virus vaccines. PCR and indirect immunofluorescence (IFA) assays were used. These isolates were propagated in chicken embryo fibroblasts obtained from a specific pathogen free breeder flock. PCR analysis of all 12 isolates resulted in the amplification of the 291-bp REV long-terminal repeat region (LTR). The subtype of the REV isolates was determined by IFA using REV-specific monoclonal antibodies. Finally DNA sequence analysis was done and it was concluded subtype 3 was the most common subtype of REV in different geographical regions in the United States.

Ongor and Bulut (2011) investigated the presence of ALV and REV in neoplastic cases observed in breeder hens older than 20 weeks. Tumour samples were examined by PCR combined with primer sets specific for ALV and REV. It was found that the tumours were REV positive. This was the first report on occurrence of REV infection in Turkey.

Mahabubulalam (2012) detected MDV in feather follicles, liver, spleen and sciatic nerve from infected chickens by PCR. All the tested samples were positive for MDV by PCR.

Cao *et al* (2013) used PCR for detection of MD and RE in formalin-fixed paraffin-embedded (FFPE) tissues and for MD in tissues only preserved in 10% neutral buffered formalin. MDV and RE virus proviral DNA were detected in FFPE tissues stored for over 20 years and MDV in tissues only preserved in formalin for up to 6 months. Finally it was concluded that PCR of formalin-fixed and FFPE tissues was simple and valuable tool that could be used to identify MD and RE infection

Jiang *et al* (2014) observed REV infection in Mallard duck in China. REV strain, DBYR1102, was first isolated and identified by PCR, indirect immunofluorescence assay and electron microscopy. The gp90 gene and complete LTR of DBYR1102 were amplified and sequenced. Phylogenetic analysis indicated that this REV strain DBYR1102 was closely related to strain HLJR0901 from northeastern China, the prairie chicken isolate APC-566, and REV subtype III, represented by chick syncytial virus and distantly related to two other subtypes of REV, 170A and SNV. This study suggested occurrence of REV in the wild birds of China and also demonstrated the potential role of wild waterfowl in REV transmission.

Sebelgy *et al* (2014) detected RE provirus DNA by PCR using LTR specific primers from tumours sample which were collected from broiler breeder flocks of 30-40 weeks of age. Histopathology of liver tumour tissue showed reticular cell infiltration replacing hepatic parenchyma. Amplified PCR product revealed genetic similarity to REV-LTR in MDV. SPF chicks were experimentally inoculated with liver homogenate of REV positive sample were housed for 8 weeks. Microscopic examination of liver and spleen showed characteristic reticular cell infiltration.

Gopal *et al* (2015) detected REV in suspected tissue sections and it was further confirmed by PCR. Due to absence of positive control for PCR, DNA isolated from immunohistochemistry positive tissue sections were utilized. Tissue samples were screened by 5’LTR-PCR. The PCR products were further confirmed by sequencing and BLAST analysis.

III. Multiplex PCR

Gopal *et al* (2012) detected the avian oncogenic viruses in poultry layer farms and turkeys by multiplex PCR. The histopathology-positive tissue sections were used and avian oncogenic viruses were confirmed by immunohistochemistry using virus-specific antibodies. The viruses belonging to MDV, ALV and REV were isolated in a cell culture system and confirmed by immunofluorescence using virus-specific antibodies. Multiplex PCR was found to be useful technique for rapid diagnosis of avian oncogenic viruses and detection of multiple infections of avian oncogenic viruses under field conditions.

Wang *et al* (2014) studied avian leukosis virus (ALV), reticuloendotheliosis virus (REV) and Marek's disease virus (MDV) differential diagnosis using oligonucleotide microarray. Particular DNA sequences were recognized using specific hybridization between the DNA target and probe on the microarray. Multiplex PCR was also used. The oligonucleotide microarray, was an easy-to-use, high-specificity, high-sensitivity and extendable assay for rapid differential diagnosis of avian oncogenic viruses.

Zeng *et al* (2015) used GeXP multiplex PCR for simultaneous detection of eight immunosuppressive chicken viruses i.e MDV, ALV – A/B/J, REV, IBDV, CIA and avian reovirus. It was concluded that GeXP multiplex PCR assay was a high-throughput, sensitive and specific method for the detection of these immunosuppressive viruses, and can be used for differential diagnosis and molecular epidemiological surveys.

4) Hot Spot PCR

Borenshtein and Davidson (1999) used two step hot spot-combined PCR assay for the identification and characterization of recombinant viruses (RM1) in Marek's disease and retrovirus co-infections. It was observed that hot spot-combined PCR as an effective method to detect the retroviral insert in RM1, the MDV integration site and the insert orientation. For confirmation of the results the herpesvirus and retrovirus chimeric PCR products sequencing was also done.

Davidson and Borenshtain (2001) studied co-infections of MDV, REV, ALV, ALV-J lead to retroviral long terminal repeat (LTR) integration into MDV. Quarter of the tumour-bearing commercial chicken and turkey flocks carried a mixed MDV and retrovirus infection. Multiple virus sequences were detected by PCR. Hot spot-combined (HS-cPCR) assay was also conducted that contained chimeric MDV and retroviral LTR molecules. Southern blotting was also done. This results indicated integration of retroviral LTR into MDV.

CHAPTER III

MATERIALS AND METHODS

3.1 Source of samples

The present study was conducted on cases of avian neoplasms recorded in birds presented for diseases diagnosis in the Poultry Disease Diagnostic Laboratory, Department of Veterinary Pathology, GADVASU, Ludhiana from 1st August 2015 to 31st January 2016.Tentative diagnosis was made on the basis necropsy findings.

3.2 Necropsy examination

Detailed examination of birds were conducted and a tentative diagnosis of neoplastic conditions was made. Gross lesions of affected organs/tissues were recorded. Samples were collected from affected organs i.e. liver, spleen, heart, kidneys, lungs, brain, proventriculus and peripheral nerves in 10% neutral buffered formalin for histopathology and immunohistochemistry. In addition samples from affected organs were collected and stored at -80°C for molecular detection of avian oncogenic viruses.

3.3 Collection and analysis of samples

3.3.1 Histopathology

Samples preserved in 10% buffered formalin were washed in running water, dehydrated in ascending grades of alcohol, then cleared in acetone and benzene and embedded in paraffin at 58°C. 5-6µm paraffin section were cut and placed on glass slides and stained using routine haematoxylin and eosin (H and E) staining technique for routine histopathology (Luna 1968).

3. 3.2 Immunohistochemistry

Paraffin tissue sections

For immunohistochemical studies 4-5 µ thick paraffin embedded tissue sections were cut and mounted on Superfrost/Plus, positively charged microscopic slides (Fisher Scientific, USA). The slides were then kept on hot plate to melt the paraffin at 60° C for 30 minutes and stored till further use.

Polyclonal antiserum

Primary antibodies used in this study were procured from Charles River Laboratories (USA). The lyophilized antibodies were reconstituted in 1 ml of sterile deionized water as per manufacture's instructions. Antibodies used were of chicken origin polyclonal Marek's disease antiserum, Reticuloendotheliosis antiserum.

The antibodies were diluted to 1:100, 1:500, 1:1000, 1:5000 and 1:10000 in PBS (Ph 7.2-7.6) for standardizing working dilutions in order to localize oncogenic viruses in tissue sections. The following dilutions were standardized and used for present study:

- Marek's disease antiserum -1:5000
- Reticuloendotheliosis antiserum-1:10000

Antigen/Epitope retrieval

Heat induced epitope retrieval (HIER) was employed using EZ antigen retrieval solutions by EZ-Retriever ™ System (Table 1) as per instructions of the data sheet (BioGenex Laboratories Inc., San Ramon, California, USA).

Table 1: Antigen retrieval protocol used for HIER in Immunohistochemistry

S. No.	Name of AR solution used	Dilution used	Temp. & Time
1.	EZ-AR™ Common Solution 5 X Concentrated	Five-fold dilution in deionized water	70°C- 10min.
2.	EZ-AR™ 3 Solution 10 X Concentrated	Ten-fold dilution in deionized water	95°C- 10min.

Immunohistochemical staining protocol

Immunohistochemical staining was performed by using Vectastain ABC Kit (Vector laboratories, Burlingame, USA) as per manufacturer's instructions. The sections were dewaxed and rehydrated by dipping in EZ-AR™ Common Solution (BioGenex Laboratories Inc., San Ramon, California, USA), and heating at 70^{0} C for 10 minutes in EZ-Retriever ™ System (BioGenex Laboratories Inc., San Ramon, California, USA) and subsequent antigen retrieval either by heat was performed in EZ-

AR^{TM} 3 Solution at 95^0 C for 10 minutes (Table 1) in EZ-Retriever^{TM} System (BioGenex Laboratories Inc., San Ramon, California, USA). Following HIER the sections were allowed to cool and brought to room temperature. Then were given three washing in PBS (pH 7.2-7.4) of 3 minutes each. Sections were encircled with hydrophobic pen. The endogenous peroxidase was quenched with a Bloxall solution (Vector laboratories, Burlingame, USA) for 15 min at room temperature in humid chamber, followed by thrice washing with PBS for 3 min each. The sections were then incubated with 1:10 dilution of a normal goat serum (Vector Laboratories, Burlingame, USA) mixed with power block (1 drop in 1 ml) (Biogenex Laboratories Inc., San Ramon, California) to block non-specific protein binding for 60 min at room temperature in humidified chamber. Afterwards the sections were incubated with reconstituted primary antibody at 4°C over night (polyclonal MD antiserum 1:5000, polyclonal RE antiserum 1:10000).

The sections were then given three washing in PBS for 3 minutes each, followed by incubation with secondary biotinylated anti-chicken IgG (Vector Laboratories, Burlingame, USA) at a concentration of 1:400 for both MDV and REV for 30 min at room temperature in humidified chamber and three washings with PBS for 3 min each. The sections were then incubated in Vectastain ABC reagent (Vector laboratories, Burlingame, USA) for 30 min at room temperature in humidified chamber and three washings with PBS for 3 min each. The antigen-antibody-peroxidase reaction was developed with a freshly prepared 3,3 '-diaminobenzidine (DAB) solution by mixing 1 drop of DAB chromogen with 1 ml of DAB buffer supplied by the manufacturer (Vector Laboratories, Burlingame, USA) and adding 5 μl hydrogen peroxide. Sections were later washed in distilled water for 5 minutes and counterstained with Gill's haematoxylin (Merck, Germany) for 30 seconds and washed in running tap water for 5 minutes. Finally the slides were dehydrated in ascending grades of alcohol, cleared in xylene, mounted in DPX and examined under microscope (BX 61, Olympus Corporation, Japan). For each antibody, a negative control was run by replacing primary antibody with PBS buffer.

3.3.3 Polymerase Chain Reaction

DNA extraction

1. DNA extraction was done from pooled samples (liver, spleen, kidney, ovary) using Qiagen DNeasy Blood and Tissue Kit. Briefly 25 mg of pooled tissue samples was taken and cut into small pieces and placed in a 1.5 ml micro centrifuge tube.
2. 180 µl of buffer ATL was added and then 20µl proteinase k (20mg/ml) was added. It was mixed by vortexing and incubated at 56°C overnight.
3. 200µl of buffer AL was added. Mixed thoroughly by vortexing and incubated at 56°C for 10 min.
4. 200µl of ethanol (99%) was added. Mixed thoroughly by vortexing.
5. Mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuged at 8000 rpm for 1 min. Flow through was discarded.
6. Spin column was placed in a new 2 ml collection tube. 500µl of buffer AW1 was added. Centrifuged for 1 min at 8000 rpm. Flow through was discarded.
7. Spin column was placed in a new 2 ml collection tube, 500µl buffer AW2 was added, and centrifuged for 3 min at 14,000 rpm. Flow through and was discarded.
8. Spin column was transferred to a new 1.5 ml or 2 ml microcentrifuge tube.
9. DNA sample was eluted by adding 150 µl buffer AE to the center of the spin column membrane. Incubated for 2 min at room temperature (15-25°C). Centrifuged for 1 min at 8000 rpm.
10. DNA sample was stored at -20°C till further use.

Conventional PCR

Conventional PCR was performed using primer specific for MDV-1 BamH1-H 132 bp tandem repeat, Proviral LTR as per Davidson and Borenshtain (1999) which amplified a target of fragment size 434bp and 291bp respectively(Table 2), using reaction mixture (Table 3) and thermal cycling conditions described in Table 4.

Table 2: Primers used for the detection of Reticuloendotheliosis and Marek's disease viruses in tissue DNA

Virus	Primer	Sequence	Gene	Product size	Reference
MDV-1	M1 (Forward)	5' TAC TTC CTA TAT AGA TTG AGA CGT 3'	MDV -1 BamH1- H 132 bp tandem repeat	434bp	Davidson and Borenshtain (1999)
	M2 (Reverse)	5'GAG ATC CTC GTA AGG TGT AAT ATA 3'			
REV	R1 (Forward)	5' CAT ACT GGA GCC AAT GGT T 3'	Proviral LTR	291 bp	
	R2 (Reverse)	5'AAT GTT GTA CCG AAG TAC T 3'			

Table 3: PCR reaction mixture used for the detection of Reticuloendotheliosis and Marek's disease viruses in tissue DNA

Components	Volume/Reaction
Master mix (2x) (Qiagen)	12.5 µl
M1 (Forward primer) 10pm / µl	1.0µl
M2 (Reverse primer) 10 pm / µl	1.0µl
Sterile water	5.5µl
Template DNA (150 ng/µl)	5µl
Total	25.0µl

Table 4: Cycling conditions used for detection of Reticuloendotheliosis and Marek's disease viruses in tissue DNA

Steps	MDV			REV		
	Temperature	Time	Cycles	Temperature	Time	Cycles
Initial Denaturation	94 °C	3 minutes	1	94°C	1 minute	1
Denaturation	94°C	45 seconds	35	94°C	1 minute	31
Annealing	55°C	40 seconds		50°C	1 minute	
Elongation	72 °C	45 seconds		72°C	1 minute	
Final Elongation	72 °C	10 minutes	1	72°C	10 minutes	1

Preparation of buffers /solutions

1 Stock solution of TBE (Tris borate EDTA) buffer -10X

Tris base	108 g
Boric acid	55g
0.5M EDTA (pH 8.0)	9.38g
DDW to make volume	1 litre

2 Working buffer (1X TBE)

10X TBE	100 ml
DDW to make volume	1 litre

3 1.5% Agarose gel (molecular biology grade)

The gel was prepared in working TBE (1x) with ethidium bromide @0.5µg/ml

Analysis of PCR Products

The PCR product was analyzed by running on agarose gel in 1X TBE buffer at 70 V for 1 hr. Along with the test samples Gene Ruler DNA ™ ladder 100 bp was also run.

Visualization of gels

Agarose gels were visualized under Geldoc (BIO-RAD) photographed and analysed with the same software.

Multiplex PCR

Multiplex PCR was performed using primer specific for meq, p27, LTR as per Gopal *et al* (2012) which amplify a target of fragment size 856 bp, 613bp and 204 bp respectively (Table 5) using reaction mixture (Table 6) and thermal cycling conditions described in table 7.

Table 5: Targets of primers used in multiplex PCR and their product size

Virus	Gene	Product Size (bp)
MDV	Meq	856
ALV	p27	613
REV	LTR	204
Chicken DNA (Control)	β-actin	396

Table 6: Reaction mixture for multiplex PCR

Components	Volume/Reaction
10X PCR Buffer	2.5 µl
dNTP	1.0 µl
Meq (Forward primer) 25pm / µl	1.0 µl
Meq (Reverse primer) 25 pm / µl	1.0 µl
p 27 (Forward primer) 25pm / µl	1.0 µl
p 27 (Reverse primer) 25 pm / µl	1.0 µl
LTR (Forward primer) 25pm / µl	1.0 µl
LTR (Reverse primer) 25 pm / µl	1.0 µl
β actin (Forward primer) 25pm / µl	1.0 µl
β actin (Reverse primer) 25 pm / µl	1.0 µl
Taq polymerase (5U/µl)	0.75 µl
DNA (150ng/µl)	5 µl
Sterile water	7.75µl
TOTAL	**25 µl**

Table 7: Cycling conditions for multiplex PCR

Steps	MDV		
	Temperature	**Time**	**Cycles**
Initial Denaturation	94 °C	5 minutes	1
Denaturation	94°C	45 seconds	30
Annealing	55°C	45 seconds	
Elongation	72 °C	45 seconds	
Final Elongation	72 °C	7 minutes	1

Hot Spot –Combined PCR (HS- c PCR)

The system included two amplification stages:

1. Amplification of the MDV-Rs fragment representing one of the hot spots for retrovirus integration, using the MDV-Rs homologous primer set (combination 1, Table 8).

2. Amplification of the stage I PCR products in six PCR tubes, each using a heterologous primer set as detailed in Table 8. The MDV and REV-LTR homologous combinations (no. 1 and 2, Table 8) were included as positive controls, while combinations no. 3–6 (Table 8) were used to detect integrants.

Table 8: Primer sets used in HS- cPCR

Combination no.	Primer set type	Primers	Product type
1	Homologous	Rs1+Rs2	MDV (Rs fragment)
2	Homologous	R1+R2	REV (LTR fragment)
3	Heterologous	Rs1+R1	MDV integrated LTR
4	Heterologous	Rs1+R2	MDV integrated LTR
5	Heterologous	Rs2+R1	MDV integrated LTR
6	Heterologous	Rs2+R2	MDV integrated LTR

Table 9: Oligonucleotide primers used in HS-cPCR

<table>
<tr><th>Virus</th><th>Amplicon</th><th>Primers</th><th></th></tr>
<tr><td rowspan="2">MDV</td><td rowspan="2">BamH1-H, D
132 bp repeat</td><td>M1(direct)</td><td>5' TAC TTC CTA TAT AGA TTG AGA CCG T</td></tr>
<tr><td>M2 (reverse)</td><td>5' GAG ATC CTC GTA AGG TGT AAT ATA</td></tr>
<tr><td rowspan="2">MDV</td><td rowspan="2">Internal repeat
Short -Rs</td><td>Rs1(direct)</td><td>5'GCC TGC AGT GCC ACG TCA AGG GAA GGG C</td></tr>
<tr><td>Rs2(reverse)</td><td>5'GCG GTA TGA GAT GCA CG</td></tr>
<tr><td rowspan="2">REV</td><td rowspan="2">LTR</td><td>R1 (direct)</td><td>5' CAT ACT GGA GCC AAT GGT T</td></tr>
<tr><td>R2(reverse)</td><td>5' AAT GTT GTA CCG AAG TAC T</td></tr>
</table>

PCR tubes were placed in DNA thermal cycler and subjected to the following conditions as shown in Table 10:

Table 10: Cycling conditions for HS-cPCR

Steps	HS- cPCR		
	Temperature	**Time**	**Cycles**
Initial Denaturation	94 °C (1 cycles)	1 minute	1
Denaturation	94°C (31cycles)	1 minute	31
Annealing	55°C (31cycles)	1 minute	
Elongation	72 °C (31cycles)	1 minute	
Final Elongation	72 °C	10 minutes	1

The HS-cPCR products of the second amplification were separated by gel electrophoresis in 1.5 % agarose gels and stained with ethidium bromide and visiualized under Gel doc.

CHAPTER 1V

RESULTS AND DISCUSSION

The present study was conducted on 1619 birds presented for post mortem examination to the Poultry Disease Diagnostic Laboratory of the Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. During the study period from 1st August 2015 to 31st January 2016, a total of 103 birds out of 1619 birds examined on necropsy showed tumours/tumour like swelling in visceral organs. Out of these 103 suspected cases of neoplasms, samples were collected from 34 cases which formed the basis of the present study.

4.1 Prevalence of avian neoplasms on the basis of necropsy

During the study period a total of 1619 birds were presented for disease diagnosis. On the basis of necropsy, 103 birds showed tumours/tumour like swelling in visceral organs. Therefore the prevalence of avian neoplasms in the present study on the basis of necropsy examination was found to be 6.36 %. Contrary to the present study, Verma *et al* (1987 and 1989) reported 44.08 % and 49.30 % sero-prevalence of MD in Arunachal Pradesh and Tripura. Dave (1989) observed 12.30 to 30.09% mortality due to acute MD. Rahman *et al* (2003) recorded 1.09% mortality in layer farms due to MDV and ALV infections. Moshira *et al* (2016) reported 40.2% prevalence of reticuloendotheliosis in birds having visceral tumours. In the present study, out of 103 cases, 52 cases were observed in layers, 39 cases in parent broilers and 12 cases were recorded in the quails. Maximum cases (101/103) were observed in adult birds, two cases were observed in birds of 18-24 weeks of age and one case was observed in 12-18 week old bird. Musa *et al* (2013) have also reported MD in 10 - 20 weeks old chickens.

4.2 Gross lesions in avian neoplasms

Grossly in the present study the neoplasms were recorded in liver, spleen, kidneys, ovary, heart, lungs, proventriculus and intestine. The gross lesions recorded in visceral organs of the affected birds are depicted in Table 11. Liver was involved in most of the cases (30/34). The gross lesions observed in the liver of the affected bird included congestion, petechial haemorrhage, paleness and mottling of liver along

with, either diffuse enlargement, enlargement with varying sized embedded foci or enlargement with varying sized greyish-white nodular lesions (Fig.1-3). Spleen was involved in 22 cases and showed mottling with either diffuse enlargement or enlargement with greyish-white foci of varying size (Fig. 4 & 5).

Table 11: Gross lesions observed in visceral organs of the affected birds

Organ affected	Lesions recorded	No. of Cases
Liver	Congestion, hemorrhages, mottling of liver along with diffuse enlargement and raised or embedded greyish-white nodules.	30
Spleen	Diffuse enlargement and/or nodular lesions, along with mottling and necrotic foci.	22
Kidney	Diffuse enlargement along with greyish-white foci.	10
Ovary	Enlargement and nodular lesions.	9
Intestine and Mesentry	Nodular lesions.	5
Lung	Greyish-white foci.	3
Proventriculus	Thickened mucosa with nodular lesions, hemorrhages and ulceration.	3
Peripheral Nerves	Enlargement of sciatic nerves.	2

Kidneys were involved in 10 cases and showed either diffuse enlargement or enlargement with embedded greyish-white foci (Fig. 6). Ovary was involved in 10 cases and also showed either diffuse enlargement or enlargement with varying sized greyish-white nodular lesions (Fig. 7). In one of the case, nodular lesions were observed in ovary as well as mesentery (Fig. 8). Intestine was involved in five cases and showed similar type of varying sized nodular lesions. In addition, there was diffuse involvement of spleen, intestine and ovary in two cases. Lungs were involved in three cases and revealed congestion along with greyish-white foci. Proventriculus was involved in three cases and showed diffuse thickening of the mucosa along with hemorrhages, ulceration and nodular lesions. Peripheral nerves were examined in all

the cases and mild enlargement of sciatic nerves was observed in two cases only. Gross lesions were not observed in brain.

Similar type of gross lesions have been described earlier in avian oncogenic virus infections. Ratnamohan *et al* (1980) observed multiple cream coloured nodules in the intestine, liver and spleen of a reticuloendotheliosis infected hen. Rao and Sarma (1984) detected single or multiple nodules in liver, spleen, kidney ovary, proventriculus, bursa, heart, nerves, skeletal muscles and eyes of layer flock suffering from MD. Okoye *et al* (1993) observed neoplastic nodules or grey foci in the internal organs especially the liver, intestines and spleen in a turkey flock and pullet flocks infected with reticuloendotheliosis. Narang *et al* (2003) observed small to large sized greyish-white tumours on liver, spleen, kidneys, heart, lungs and thickened sciatic nerves in layer flocks having MDV infection. Cortes and Cardona (2004) also observed enlarged liver, spleen, kidneys and gonads during experimental study on MD, and found yellow to grey friable tumours in liver, spleen, kidneys, gonads, proventriculus heart and lungs. Singh *et al* (2012) reported that MD was most common lympho-proliferative disease of chickens resulting in development of tumours in visceral organs. Recently Abreu *et al* (2016) described tumorous lesions in liver and ovary, enlarged kidney and spleen in birds suffering from Marek's disease. Hughes *et al (*2016) detected diffusely enlarged spleen, kidneys and liver, with multifocal creamish foci in a turkey flock having Marek's disease. Moshira *et al* (2016) diagnosed reticuloendotheliosis in chickens and observed multiple neoplastic nodules in the livers and spleen along with enlargement of kidneys.

4.3 Histopathological lesions in avian neoplasms

In the present study, the etiology of avian neoplasms could not be ascertained on the basis of gross lesions. So histopathology was done to differentiate the avian oncogenic virus infections**.** The microscopic lesions observed in different organs in the present study are presented in Table 12.

Table 12: Microscopic lesions observed in visceral organs showing tumours

Organ	Lesions
Liver	Focal and or diffuse multifocal infiltration of pleomorphic cells.
Spleen	Diffuse infiltration of pleomorphic cells leading to disruption of architecture.
Kidney	Diffuse or multifocal infiltration of pleomorphic cells in between tubules along with congestion and necrosis of tubular epithelial cells.
Ovary	Diffuse infiltration of pleomorphic cells.
Intestine	Infiltration of pleomorphic cells in tunica mucosa, submucosa, muscularis and serosa.
Lung	Infiltration of pleomorphic cells.
Proventriculus	Focal to diffuse infiltration of pleomorphic cells in mucosa, proventricular glands and serosal layer.
Heart	Diffuse infiltration of pleomorphic cells between the myocardial fibers leading to disruption of architecture.
Brain	Gliosis, satellitosis, neuronophagia and perivascular cuffing.
Nerve	Odema and focal accumulation of cells.

Liver

Microscopically in most of the cases, liver showed either focal or multifocal infiltration of pleomorphic cells replacing hepatic parenchyma (Fig. 9 & 10). The infiltration of pleomorphic cells was also detected around blood vessels. The infiltrating cells showed mitotic figures and large degenerating macrophages suggestive of MD cells. Other lesions recorded in liver were congestion and necrosis of the hepatocytes.

Spleen

The spleen in different cases also showed focal to diffuse infiltration of pleomorphic cells leading to disruption of architecture of spleen (Fig.11& 12).

Kidneys

Kidneys showed congestion and necrosis of the tubular epithelial cells along with diffuse infiltration of pleomorphic cells between the tubules (Fig.13 & 14).

Ovary

Ovary also showed infiltration of pleomorphic cells in majority of the cases along with focal accumulation of monomorphic cells (Fig.15 & 16) in two cases.

Intestine

Intestine showed massive infiltration of pleomorphic cells in the tunica mucosa, submucosa (Fig. 17), muscularis and serosal layer. In one case, the infiltration of pleomorphic cells extended upto the mesentery (Fig.18).

Lungs

Lungs showed diffuse infiltration of pleomorphic cells in majority of the cases along with focal infiltration of monomorphic cells (Fig.19) in two cases.

Proventriculus

Proventriculus also showed infiltration of pleomorphic cells in mucosa, proventricular glands (Fig. 20 & 21), muscularis and serosal layers. In one case, proventriculus showed focal infiltration of monomorphic cells.

Heart

Heart showed degeneration and necrosis of the myofibrils along with diffuse infiltration of pleomorphic cells between the myocardial fibers leading to disruption of myocardium (Fig. 22 &23). The infiltration of pleomorphic cells was also seen in perivascular areas.

Nervous tissues

Brain revealed gliosis, satellitosis, neuronophagia and perivascular cuffing (Fig. 24 & 25). Sciatic nerves showed edema and focal aggregation of cells (Fig.26).

Earlier workers have also described infiltration of neoplastic cells in tissue sections of visceral organs of birds suffering from avian oncogenic virus infections. Payne and Biggs (1967) reported that lymphomatous lesions of MD in the visceral organs consisted of diffusely proliferating small to medium lymphocytes, lymphoblasts and primitive reticular cells. Fujimoto *et al (*1971) described cell proliferation in the interlobular connective tissues, especially around the small blood vessels in the liver, and in the spleen in birds having MDV infection. The proliferation was also seen around the capillary sheathed arteries, in the ovary, adrenals and kidneys proliferation started around the capillary or small arterioles in various tissues and extended into the adjoining tissues. Similarly, in the present study

infiltration of pleomorphic cells was seen around blood vessels in liver and heart as reported by Neuhybel *et al* (1986).Grimes and Purchase (1973) observed multiple, irregularly shaped perivascular foci of mononuclear, lymphoreticular cells in liver and other organs with tumour involvement in birds suffering from reticuloendotheliosis. Paul *et al* (1976) studied reticuloendotheliosis in a turkey flock and observed lymphoreticular cells in the liver, spleen, heart, intestines, and peripheral nerves. Kobayashi *et al* (1986) observed diffuse infiltration and proliferation of lymphoid cells of varying size in liver, spleen duodenum, small intestine proventriculus and kidney in MD outbreaks. Mohapatra (1993) reported focal or diffuse infiltration of pleomorphic lymphocytes in Marek's diseases in quails. Gurel *et al* (2003) observed lymphoid cell infiltration of various sizes particularly in liver and to lesser extent in kidneys, spleen, proventriculus and sciatic nerves of MDV infected birds. On the other hand, contrary to our findings, Gopal *et al* (2015) observed homogenous monomorphic lymphoid cell infiltrationin reticuloendotheliosis. Moshira *et al* (2016) also recorded multiple neoplastic nodules of homogeneous lymphocytes in the livers and spleen in a REV infected chickens flock.

Although earlier workers have reported lesions in the peripheral nerves in birds suffering from MD and RE, however, in the present study lesions in sciatic nerves were observed in two cases only (Table 11). Peripheral nerves are mainly involved in classical form of Marek's disease and not in visceral form of Marek's disease. Previous studies conducted in the department also suggest more prevalence of visceral form of Marek's disease with negligible lesions in nerves (Goyal 2004, Kaur 2010, Lawhale 2013, Mitra *et al* 2013).

4.4 Immunohistochemical localization of avian oncogenic viruses in tissue sections

Tissue samples from all the cases were subjected to imunohistochemical staining using virus specific antisera to detect the presence of various avian oncogenic viruses alone or as mixed infections as described earlier (Gopal *et al* 2012).

On the basis of immunohistochemistry, 29 cases out of the total 34 cases were found to be positive for MD, 28 cases were found positive for RE, whereas, 28 cases showed positive reactivity for both MD and RE. The results of immunohistochemistry have been summarized in Table 13. In the present study, on the basis of immunohistochemistry the prevalence of MD was found to be 85.3% (29/34), that of RE was

82.3% (28/34) and the concurrent prevalence of both MD and RE was found to be 82.3% (28/30). There was no case which showed positive reactivity for RE alone, and all the cases which were positive for RE were also positive for MD suggesting mixed infection of MD and RE under field conditions.

Table 13: Immunoreactivity and prevalence of MD and RE in the affected birds

IHC Findings			
Case	**MD**	**REV**	**MD+ REV**
N= 34	29	28	28
Prevalence	85.3%	82.3%	82.3%

4.4.1 Immunoreactivity of MD in avian neoplasms

Moderate to strong reactivity for MDV antigen was detected in the cytoplasm as well as nucleus of the infiltrating neoplastic cells in the different visceral organs. Similarly, Cho *et al* (1999) demonstrated MDV particles with in necrotic lymphoblasts with the help of electron microscopy and immunohistochemistry in 1 day SPF chicks inoculated with MDV. Gopal *et al* (2012) detected positive reactivity for MDV in the nuclei of the lymphoid cells in the liver of birds naturally infected with MDV. In addition, in the present study, mild positive reactivity for MD was also detected in the hepatocytes, tubular epithelial cells of the kidney, myocardial fibers, and endothelial cells of the blood vessels and neurons of the brain (Fig. 27). Similarly, positive reactivity of MDV antigens has been reported earlier in feather follicular epithelium, kidney tubular epithelial cells and lymphocytes of bursa in MDV affected chicks (Calnek and Hitchner 1969). The positive reactivity for MD in infiltrating neoplastic cells in liver, spleen, kidneys, heart, ovary, lungs, intestine and mesentery observed in the present study has been depicted in figures 28-34. Similarly, Kaur 2010 described positive reactivity of MD in transforming cells in the liver, spleen, lungs, kidney, ovary, mesentery and proventriculus of birds naturally infected with MDV. Although immunophenotyping of avian neoplasms was not done in the present study, Pejovic *et al* (2007) described immunophenotypic characteristics of MD tumours in chickens, and reported that lymphoma cells in MD were of CD3+ phenotype.

4. 4.2 Immunoreactivity of RE in avian neoplasms

Moderate to strong immunoreactivity for REV antigen was also detected in the cytoplasm as well as nucleus of the infiltrating neoplastic cells in the liver (Fig. 35), spleen (Fig.36), kidneys (Fig.37), heart (Fig.38), ovary, lungs (Fig. 39), intestine (Fig. 40), and mesentery in different cases. Earlier workers have also described positive reactivity of REV in both the cytoplasm as nuclei of cells infected with REV (Santos *et al* 2009, Kaur 2010 and Gopal *et al* 2012). As observed in present study, Santos *et al* (2008) also reported that immunohistochemistry was a potent diagnostic tool for detection of REV infections, as microscopic lesions of RE cannot be differentiated from lesions caused by lymphoid leukosis and Marek's diseases viruses. Immunohistochemistry has been applied to differentiate avian oncogenic viruses by earlier workers. Xiu-guo *et al* (2008) used immunohistochemistry for detection of MDV and REV in broiler breeders, and concluded that differential diagnosis of MD and REV can be done by immunohistochemistry as observed in present study. Similarly, Kaur 2010, reported positive reactivity of REV in the liver, spleen, lungs and kidneys of REV infected birds, and concluded that immunohistochemistry was more sensitive technique as compared to necropsy and histopathology for diagnosis of various neoplasms caused by avian oncogenic viruses under field conditions.

4.5 Molecular Studies

In the present study MD and RE could not be differentiated on the basis of gross and microscopic lesions. The immunohistochemical localization of MDV and REV antigens suggested mixed infection of MDV and REV in field conditions. Therefore the samples were subjected to various types of polymerase chain reactions to further confirm the etiology of the avian neoplasms, and to detect mixed avian oncogenic virus infections.

4.5.1 Conventional PCR

Using specific primers for MD and REV (Davidson and Borenshtain 1999), 29 cases out of the total 34 cases were found to be positive for MDV (Table 14) with amplification of 434 bp products (Fig. 41). The primer sequences employed for MDV-1 *Bam*H1-H 132bp tandem repeat amplified the genomic sequences of pathogenic MDV-1 only as these primers differentiate between pathogenic and non-pathogenic serotype-1 MDV and vaccine viruses of MDV-serotypes 2 and 3 (Becker *et al* 1992 and Mitra et al 2013). As reported earlier, the direct primer of MDV is

located 65bp 5' to the tandem 132bp repeat and the reverse primer is 105bp downstream of the 132bp repeat. Therefore, the amplified DNA band size expected in case of a double 132bp repeat is 434bp (Silva 1992 and Mitra *et al* 2013), as observed in present study. On the other hand, only 15 cases out of total 34 cases were found to be positive for REV (Table 14) with amplification of 291 bp product (Fig. 42), which suggests proviral LTR of REV. Sebelgy *et al* (2014) also detected reticuloendotheliosis provirus DNA in tumours samples by PCR using LTR specific primers. In the present study, on the basis of conventional PCR, the prevalence of MD and REV was found to be 85.3% (29/34) and 44.12% (15/34) respectively. Further the concurrent prevalence of MD and REV was found to be 44.12% suggesting mixed infection of MD and REV. In the present study, no case was found to be positive for REV alone, and all the cases which were positive for REV were also found positive for MDV (Table14), as observed in immunohistochemical staining also. Davidson and Borenstein (1999) used PCR, and reported prevalence of 76 % and 75 % of single avian oncogenic virus infection in chicken and turkey flocks respectively, and 24% and 25 % prevalence of multiple avian oncogenic virus infections in chickens and turkeys flocks respectively.

Conventional PCR has been applied for specific diagnosis of avian oncogenic viruses by earlier workers. Zhu *et al* (1992) applied PCR and used long inverted repeats of MDV1 DNA for differentiation of oncogenic and non-oncogenic strains of MDV type 1. Fu *et al* (1993) developed rapid PCR assay specific for MDV. Bumstead *et al* (1997) developed quantitative assay for MDV which determined the number of viral genome by PCR. Kozdrun *et al* (2001) used three different reactions and primers for gene A in PCR to differentiate MDV strains belonging to serotypes 1 and 3, and also the distinction of virulent and attenuated strains within the limits of serotype 1. Mahabubulalam (2012) detected MDV by PCR in the feather follicles, liver, spleen and sciatic nerve collected from MDV infected chickens. On the other hand, Mitra *et al* 2013 used PCR for early detection of avian oncogenic viruses from blood DNA of apparently healthy chickens.

In the present study conventional PCR was found to be helpful in differential diagnosis of avian oncogenic viruses as reported earlier. Davidson *et al* (1995) studied normal and tumour-bearing birds from a total of 16 chicken and turkey flocks using PCR for differentiating between Marek's MDV and REV. Davidson *et al*

(1998) reported that PCR had advantage over other techniques for differential diagnosis of avian oncogenic viruses under natural conditions. Cao *et al* (2013) used PCR for detection of MD and RE in formalin-fixed paraffin-embedded (FFPE) tissues, and concluded that PCR of formalin-fixed and FFPE tissues was simple and valuable tool that can be used to identify MD and RE infection. Mitra *et al* 2013 detected concurrent infection of multiple oncogenic viruses, and further reported that PCR was a simple diagnostic technique for detecting latent avian oncogenic virus infections under field conditions.

Table 14: Prevalence of avian oncogenic viruses on the basis of Conventional PCR

Conventional PCR			
Total samples	**MD**	**REV**	**MD+ REV**
34	29	15	15
Prevalence	**85.3%**	**44.12%**	**44.12%**

4.5.2 Multiplex PCR

As the result of immunohistochemistry and conventional PCR suggested mixed infection of MD and REV, so the samples were subjected to multiplex PCR using standard primers for MDV (meq gene), REV(LTR) and ALV (p27 gene). On the basis of multiplex PCR, 24 out of 34 cases were found to be positive for MDV, and 15 out of 34 were positive for REV (Fig.43). In addition, two cases were found to be positive for MD, REV and ALV (Fig. 44). Thus in the present study, the prevalence of MDV, REV, ALV and concurrent MDV and REV on the basis of multiplex PCR was found to be 70.59%, 44.12%, 5.88% and 44.12% respectively (Table 15). Further, all the 15 cases which were positive for REV were also positive for MDV as observed in immnuhistochemical staining and conventional PCR.

Table 15: Prevalence of avian oncogenic viruses on the basis of Multiplex PCR

	Multiplex PCR			
Total samples	**MD**	**REV**	**ALV**	**MD+ REV**
34	24	15	2	15
Prevalence	**70.59%**	**44.12%**	**5.88 %**	**44.12%**

As observed in the present study, multiplex PCR was found to be useful for rapid differential diagnosis of avian oncogenic viruses and detection of multiple infections of avian oncogenic viruses under field conditions (Gopal *et al* 2012). Wang *et al* (2014) also used multiplex PCR for differential diagnosis of avian oncogenic viruses. Zeng *et al* (2015) used GeXP- multiplex PCR for simultaneous detection of eight immunosuppressive chicken viruses including MDV, ALV (A/B/J), REV, IBDV, CIAV and avian reo viruses. They concluded that GeXP-multiplex PCR could be used for differential diagnosis of different virus infections, and also for the molecular epidemiological surveys.

4.5.3 Hot spot combined PCR

In the present study, immunohistochemistry, conventional PCR and multiplex PCR revealed concurrent presence of MDV and REV. So Hot spot c-PCR was applied to detect the insertion of REV genome into the MDV genome using specific primers. Although, in the present study, concurrent MDV and REV infection was detected in 28/34 cases by immunohistochemistry, and 15/34 by conventional and multiplex PCR, but no insertion of REV genome into MDV genome was detected in any case by Hot spot c- PCR using primers as described earlier. Davidson *et al* 1998 conducted the survey of commercial tumour bearing birds for avian oncogenic viruses and reported that both herpesviruses and retroviruses could infect the same bird under natural conditions. Borenshtein and Davidson (1999) used two step Hot Spot-combined PCR assay for the identification and characterization of recombinant viruses (RM1) in MDV and REV co-infections. Hot Spot-combined PCR was found to be an effective method to detect the retroviral insert in RM1, the MDV integration site and the insert orientation. In addition, Davidson and Borenshtain (2001) studied co-infections of MDV, REV, ALV, ALV-J and reported retroviral LTR integration into MDV.

Diagnostic efficacy of different diagnostic techniques for detection of avian oncogenic virus infections has been summarized in Table 16. As compared to the PCR, immunohistochemistry was found to be more sensitive in detection of avian oncogenic virus infection under field conditions.

Table 16: Diagnostic efficacy of different techniques used for detection of avian oncogenic viruses

Samples	IHC			Conventional PCR			Multiplex PCR			
	MD	REV	MD+ REV	MD	REV	MD+ REV	MD	REV	ALV	MD+ REV
34	29	28	28	29	15	15	24	15	2	15
Prevalence (%)	85.3	82.3	82.3	85.3	44.12	44.12	70.59	44.12	5.88	44.12

From the study it was concluded that mixed avian oncogenic virus infections are common under field conditions. The avian oncogenic virus infections cannot be differentiated on the basis of gross and microscopic lesions. Both immunohisto-chemistry and PCR were found to be effective in differential diagnosis of avian oncogenic virus infections. Multiplex PCR was found to be effective in detection of multiple avian oncogenic virus infection under field conditions. Although in the present study, concurrent infection of MD and REV was detected in 28 cases by immunohistochemistry, and in 15 cases by PCR, but no insertion of REV genome into MDV genome could not be detected by Hot spot combined PCR.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study was conducted on 1619 birds presented for post mortem examination to the Poultry Disease Diagnostic Laboratory of the Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, from 1st August 2015 to 31st January 2016. On the basis of necropsy examination 103 birds showed tumours/ tumour like swelling in visceral organs. 52 cases were observed in layers, 39 cases in parent broilers and 12 cases were recorded in the quails. Maximum cases (101/103) were observed in adult birds, two cases were observed in 18-24 weeks old birds and one case was observed in 12-18 week old bird. Samples were collected from 34 cases which formed the basis of the present study. The prevalence of avian neoplasms in the present study on the basis of necropsy examination was found to be 6.36 %.

Grossly, tumours were recorded in liver, spleen, kidneys, ovary, heart, lungs, proventriculus and intestine. Liver was involved in 30 cases. The gross lesions in the liver were congestion, petechial haemorrhage, paleness and mottling along with either diffuse enlargement, enlargement with embedded foci or with varying sized greyish-white nodular lesions. Spleen was involved in 22 cases and showed mottling with either diffuse enlargement or enlargement with greyish-white foci of varying size. Kidneys were involved in 10 cases and showed either diffuse enlargement or enlargement with embedded greyish-white foci . Ovary was involved in 10 cases and showed either diffuse enlargement or enlargement with varying sized greyish-white nodular lesions. Intestine was involved in five cases and showed varying sized nodular lesions. Lungs were involved in three cases and revealed congestion along with greyish-white foci. Proventriculus was involved in three cases and showed diffuse thickening of the mucosa hemorrhages, ulceration and nodular lesions. Peripheral nerves mainly sciatic nerve were enlarged in two cases and gross lesions were not observed in brain.

Microscopically, focal to diffuse infiltration of pleomorphic cells was observed in liver, spleen and heart leading to disruption of the architecture of these organs. Similar type of infiltration was also detected in ovary and lungs. There was infiltration of pleomorphic cells in mucosa, submucosa, muscularis and serosal layers of proventriculus and intestines. In kidneys infiltration of pleomorphic cells was observed between the

tubules. In brain there was gliosis, satellitosis, neuronophagia and perivascular cuffing and edema and focal aggregation of cells in nerves in one case.

In the present study the etiology of avian neoplasms could not be pinpointed on the basis of gross and microscopic lesions so the samples were also screened by immuohistochemical staining, and conventional and multiplex PCR in order to detect avian oncogenic viruses in the affected tissues.

Immnunohistochemical staining revealed moderate to strong reactivity for MDV and REV associated proteins in the cytoplasm as well as nucleus of the infiltrating neoplastic cells in the different visceral organs. 29/34 cases were positive for MD, 28/34 were RE, whereas, 28 cases were positive for both MD and RE. The prevalence of MD, RE and concurrent MD and RE the basis of immunohisto-chemistry was 85.3%, 82.3% and 82.3% respectively. All the cases which were positive for RE were also positive for MD suggesting mixed infection of MD and RE.

Using specific primers of MDV and REV with the help of conventional PCR, 29 cases of MD and 15 cases of REV were found positive with amplification of 434 bp product for MD and 291 bp product for REV. In addition 15 cases were positive for both MD and REV. The prevalence of MD, REV and concurrent MD and REV on the basis of conventional PCR was 85.3%, 44.12% and 44.12%, respectively. All the cases which were positive for REV were also positive for MDV as observed in immunohistochemical staining.

As the results of immunohistochemistry and conventional PCR suggested mixed infection of MDV and REV, so the samples were subjected to multiplex PCR to detect multiple avian oncogenic virus infections. Multiplex PCR, revealed 24 cases positive for MDV infection, 15 cases positive for REV infection and 15 cases for MDV and REV mixed infection. In addition, two cases were also found positive for ALV along with MDV and REV. On the basis of multiplex PCR the prevalence of MDV and REV infection was found to be 70.59% and 44.12%, respectively. The prevalence of MDV and REV mixed infection was found to be 44.12% and mixed infection of MDV, REV and ALV was 5.88%.

Finally, hot spot combined PCR was applied to detect the insertion of REV genome into the MDV genome, but no insertion of REV genome into MDV genome could be detected in any of the confirmed cases of mixed infection of MDV and REV.

CONCLUSIONS

- In the present study the prevalence of visceral tumours on the basis of necropsy findings was found to be 6.36%.
- The MDV and REV infections cannot be differentiated on the basis of gross and microscopic lesions.
- Both PCR and IHC were found to be effective in detection of avian oncogenic virus infections under field conditions.
- Multiplex PCR was found to be a useful for rapid differential diagnosis of avian oncogenic viruses and detection of multiple infections of avian oncogenic viruses.
- However, in the present study, the insertion of REV into MDV could not detected by Hot- spot combined PCR, hence, further studies using different set of primers are required to detect the possibility of insertion of REV into MDV.
- A combination of advanced diagnostic techniques like IHC and different type of polymerase chain reactions are required for detection of avian oncogenic viruses under field conditions.

Gross lesions observed in avian oncogenic virus infections

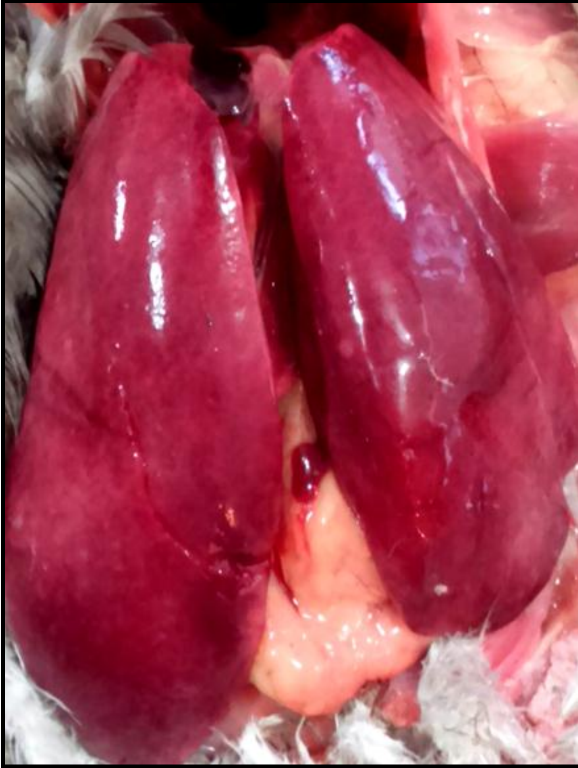

Fig 1: Liver showing diffuse enlargement

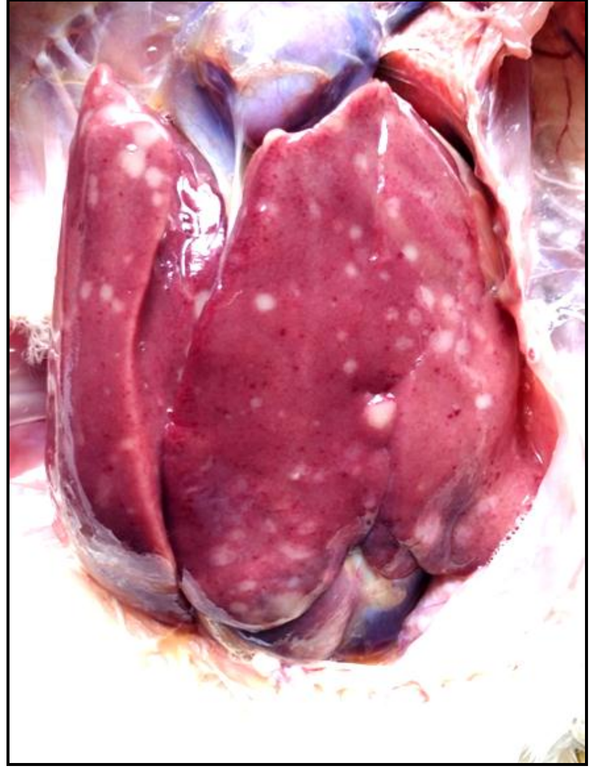

Fig 2: Diffuse enlargement of liver with embedded/ raised foci

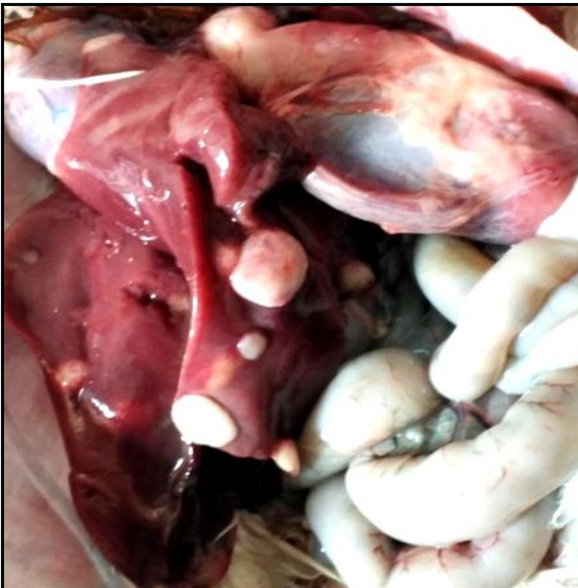

Fig 3: Liver showing varying sized raised nodular lesions

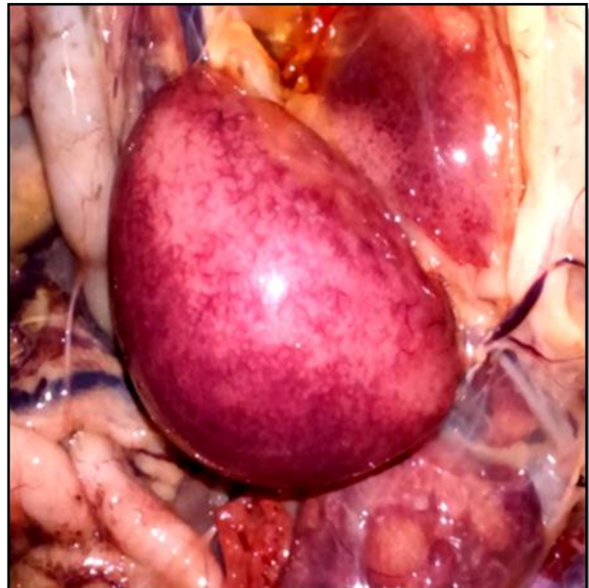

Fig 4: Mottling and diffuse enlargement of spleen

Gross lesions observed in avian oncogenic virus infections

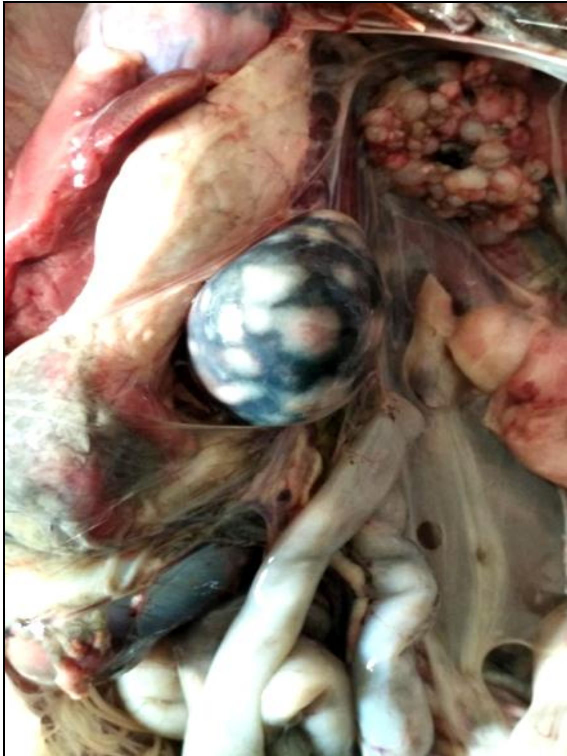

Fig 5: Spleen showing varying sized greyish- white foci

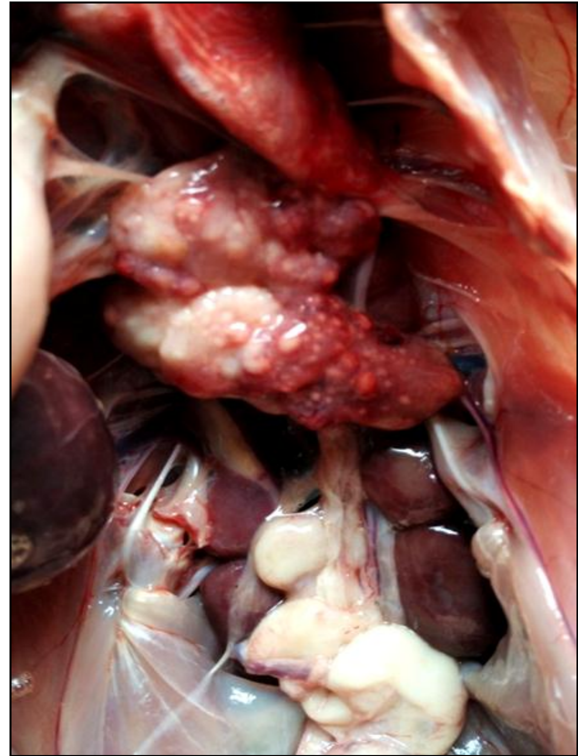

Fig 6: Enlargement of kidneys along with greyish-white foci and nodular lesions in ovary

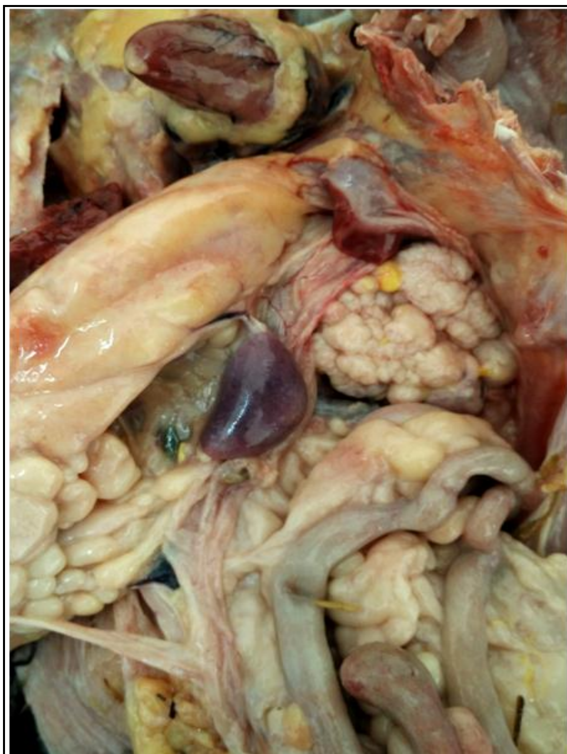

Fig 7: Nodular lesions in ovary

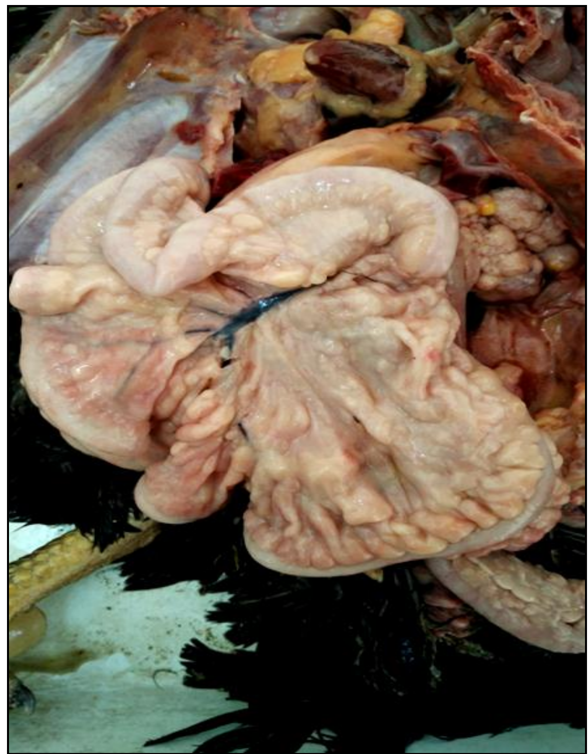

Fig 8: Nodular lesions in the mesentery and ovary

Histopathological lesions observed in avian oncogenic virus infections

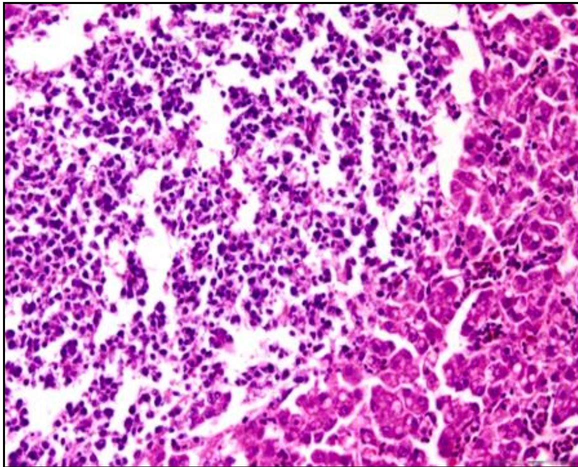

Fig 9: Microphotograph of liver showing focal aggregation of pleomorphic cells replacing the parenchyma, H&E x 40X.

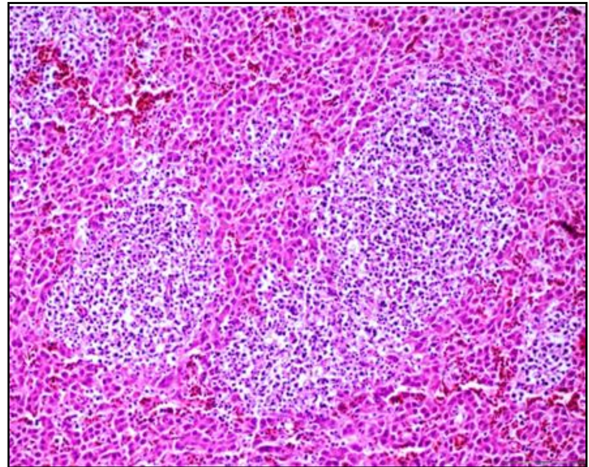

Fig 10: Liver showing multifocal aggregation of pleomorphic cells, H&E x 20X.

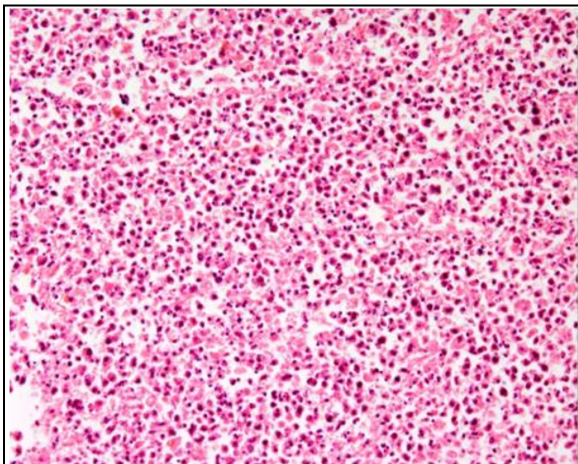

Fig 11: Spleen showing diffuse infiltration of pleomorphic cells, H&E x 40X.

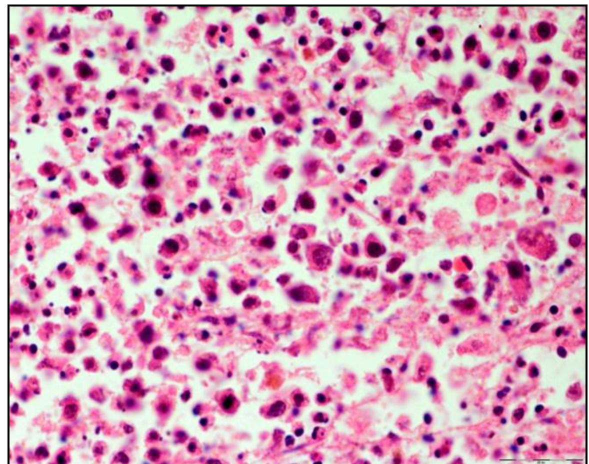

Fig 12: High power of the previous figure showing the lesions more clearly, H&E x 100X.

Histopathological lesions observed in avian oncogenic virus infections

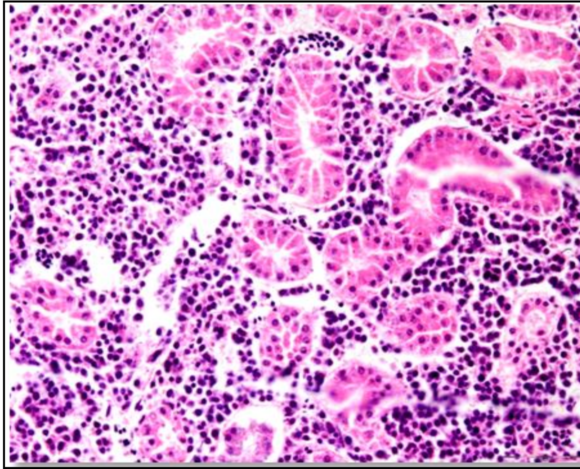

Fig 13: Kidney showing diffuse infiltration of pleomorphic cells in between the tubules, H&E x 40X.

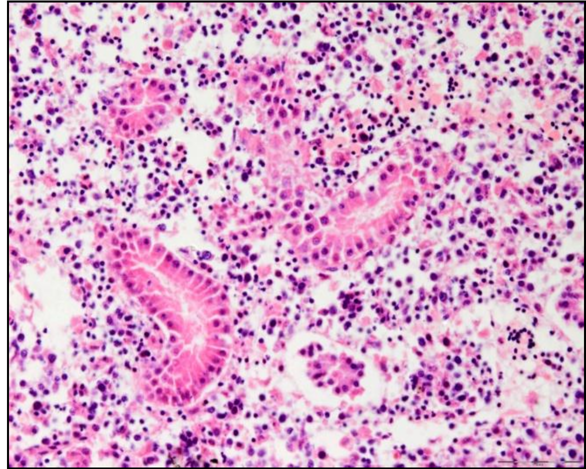

Fig 14: Similar type of lesions in kidney from another case, H&E x 40 X.

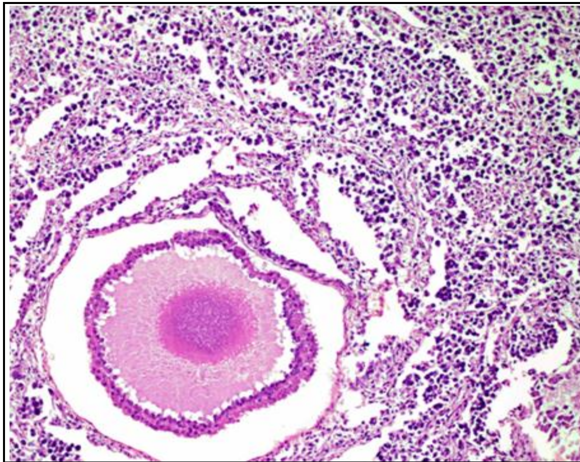

Fig 15: Diffuse infiltration of pleomorphic cells in ovary, H& E x 20X.

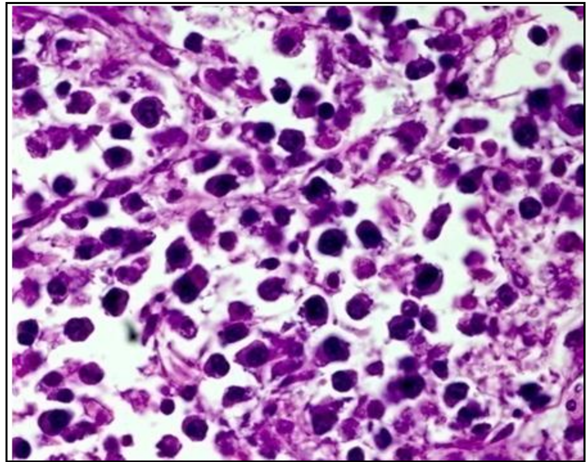

Fig 16: High power of the previous section showing the pleomorphic cells more clearly, H&E x 100X.

Histopathological lesions observed in avian oncogenic virus infections

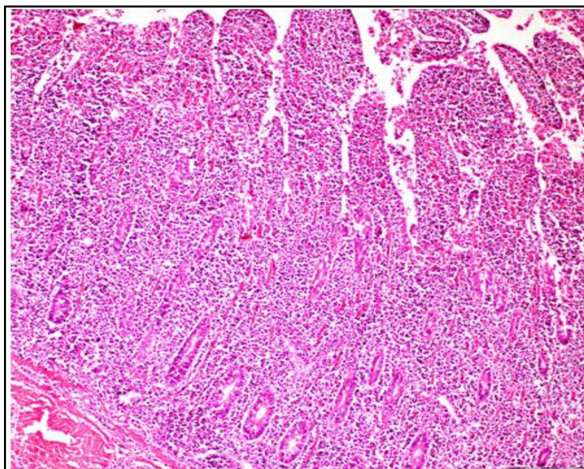

Fig 17: Diffuse infiltration of pleomorphic cells in intestine, H&E x 10 X.

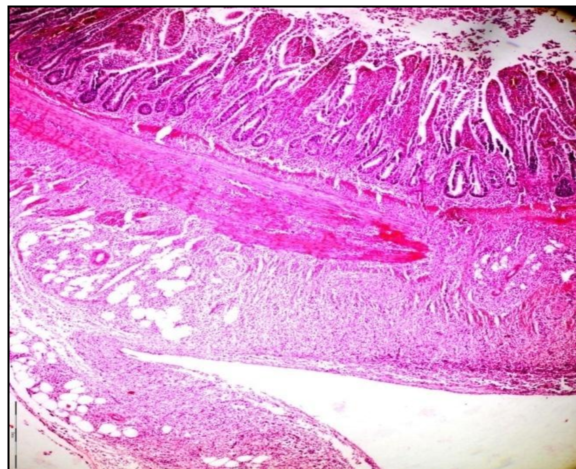

Fig 18: Diffuse infiltration of pleomorphic cells in intestine and extending up to the mesentery, H&E x 4X.

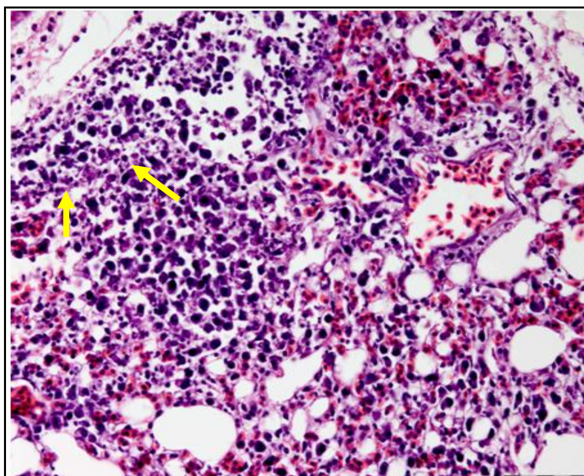

Fig 19: Infiltration of pleomorphic cells in lungs along with focal infiltration of monomorphic cells (arrows), H&E x 40X.

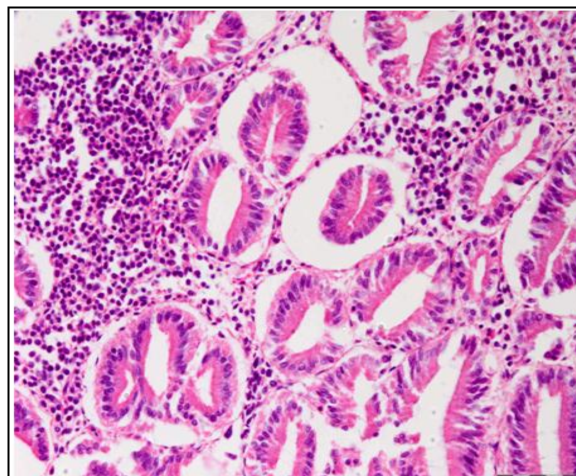

Fig 20: Diffuse infiltration of pleomorphic cells in proventriculus, H&E x 40 X.

Histopathological lesions observed in avian oncogenic virus infections

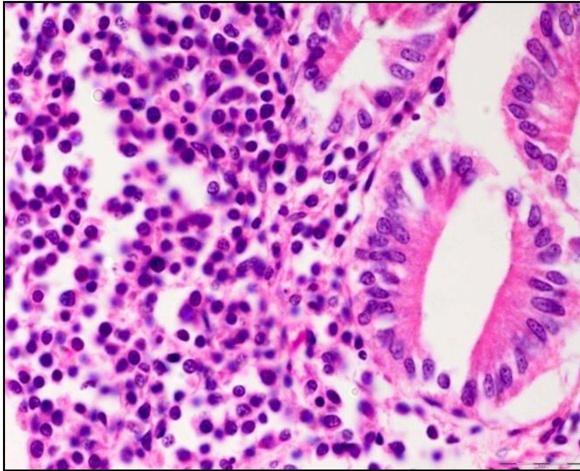

Fig 21: High power of previous figure showing the lesions more clearly, H&E x 100 X.

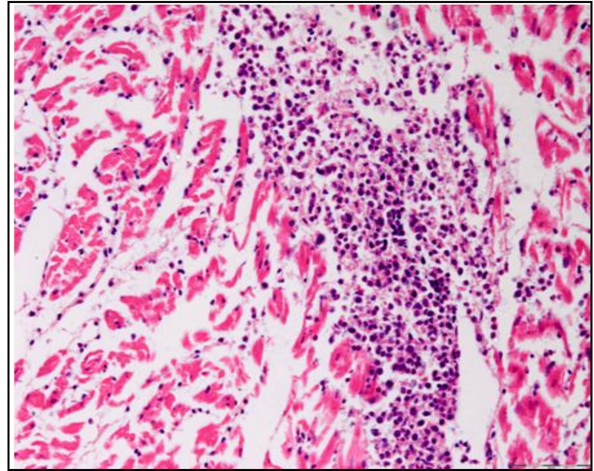

Fig 22: Infiltration of pleomorphic cells in between myocardium, H&E x 40 X.

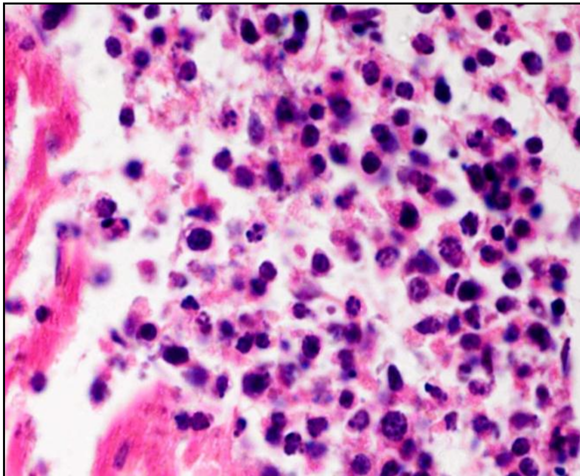

Fig 23: High power of the previous figure showing the lesions more clearly, H&E x 100 X.

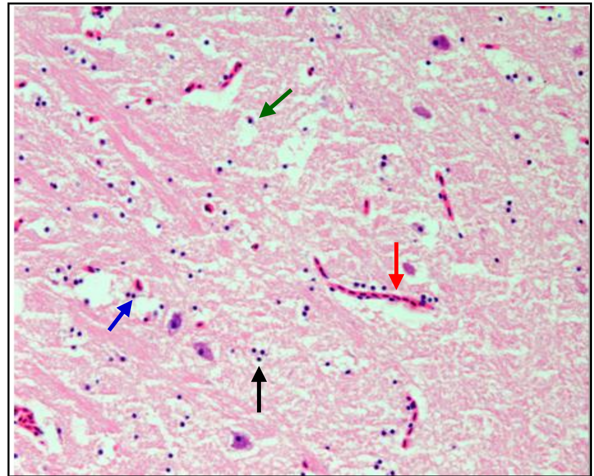

Fig 24: Brain showing, gliosis (black arrow), satellitosis (blue arrow), neuronophagia (green arrow) and perivascular cuffing (red arrow), H&E x 40X.

Histopathological lesions observed in avian oncogenic virus infections

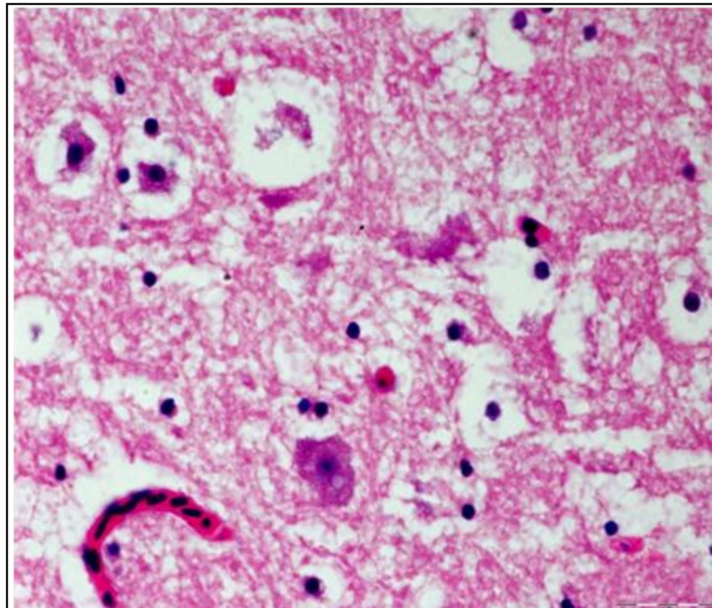

Fig 25: High power of the previous figure showing the lesions more clearly, H&E x 100 X.

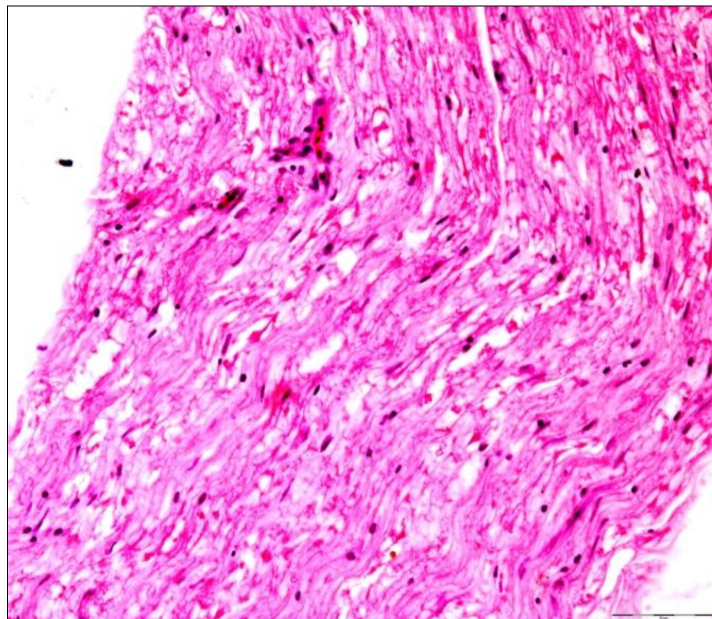

Fig 26: Nerve showing oedma and focal aggregation of cells, H&E x 40X.

Immunohistochemical detection of avian oncogenic viruses in formalin-fixed paraffin embedded tissue sections

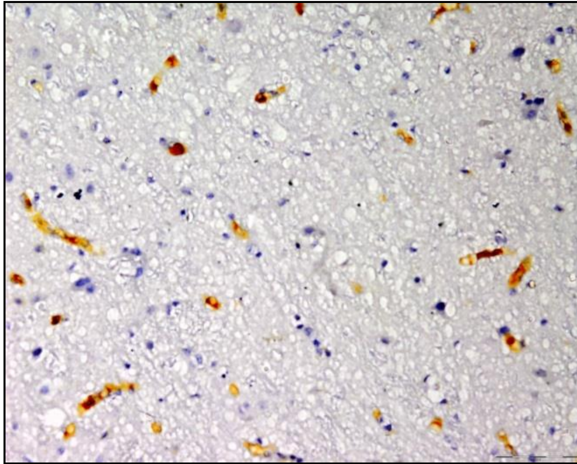

Fig 27: Cerebrum showing positive immune-reactivity for MDV antigen in the endothelial cells of the blood vessel and neurons. Vectastain ABC staining, counter-stained by Gill's haematoxylin x 40X.

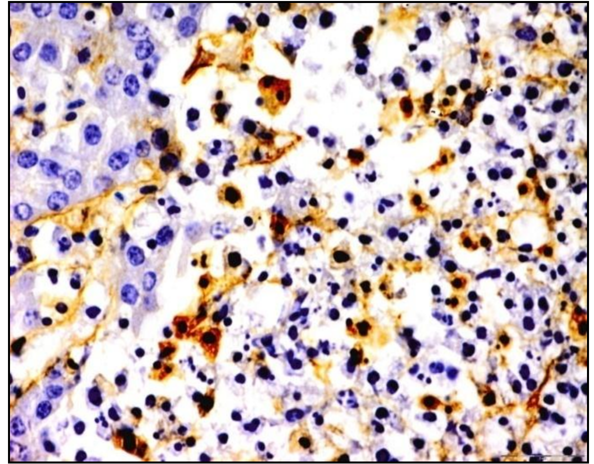

Fig 28: Liver showing positive cytoplasmic as well as nuclear immunoreactivity for MDV antigen. Vectastain ABC staining, counter-stained by Gill's haematoxylin x 100 X

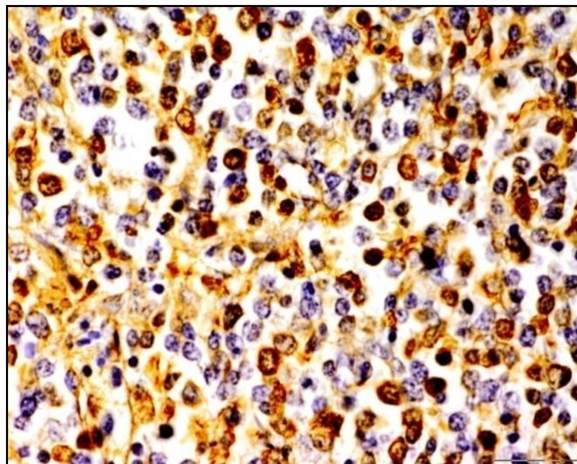

Fig 29: Spleen showing positive reactivity for MDV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x100X

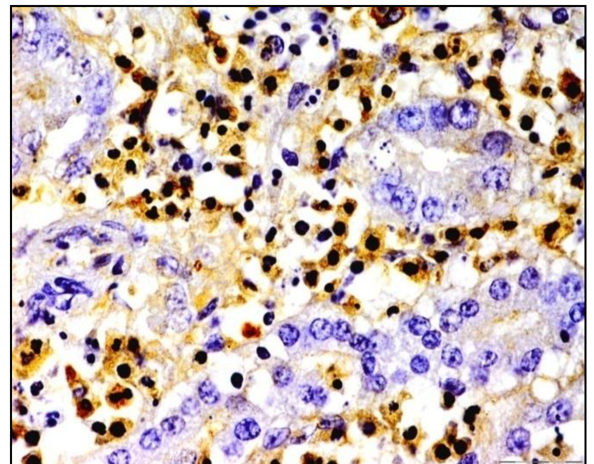

Fig 30: Kidney showing positive reactivity for MDV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 100X.

Immunohistochemical detection of avian oncogenic viruses in formalin-fixed paraffin embedded tissue sections

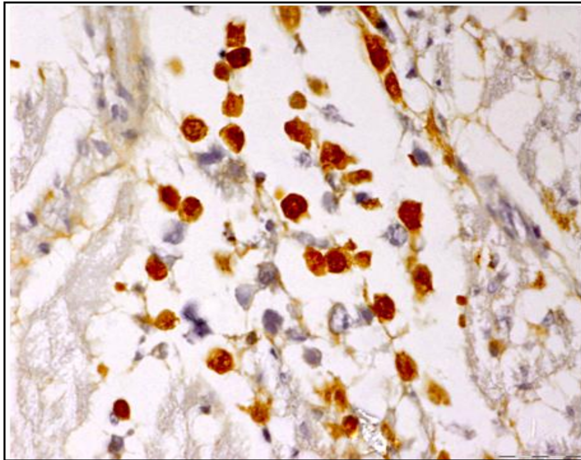

Fig 31: Myocardium showing positive reactivity for MDV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 100 X.

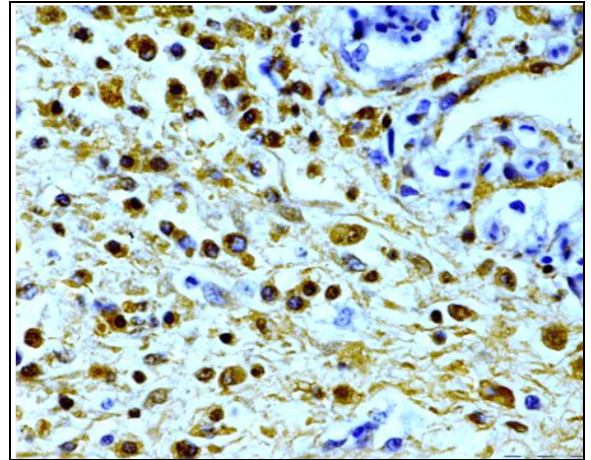

Fig 32: Lungs showing positive reactivity for MDV antigen in a combined infection of MDV, REV and ALV. Vectastain ABC staining, counterstained by Gill's haemat-oxylin x 100 X

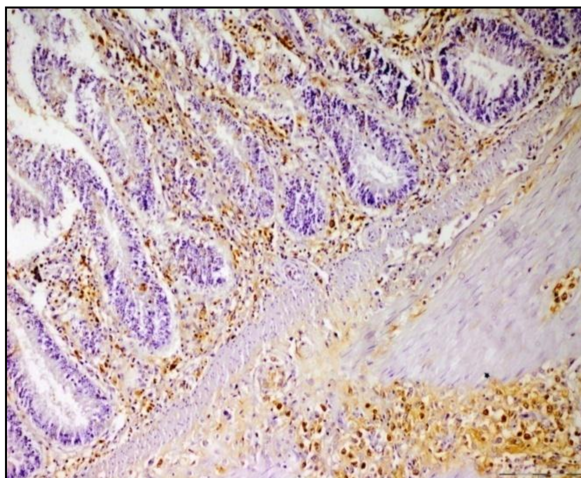

Fig 33: Intestine showing positive reactivity for MDV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 20X.

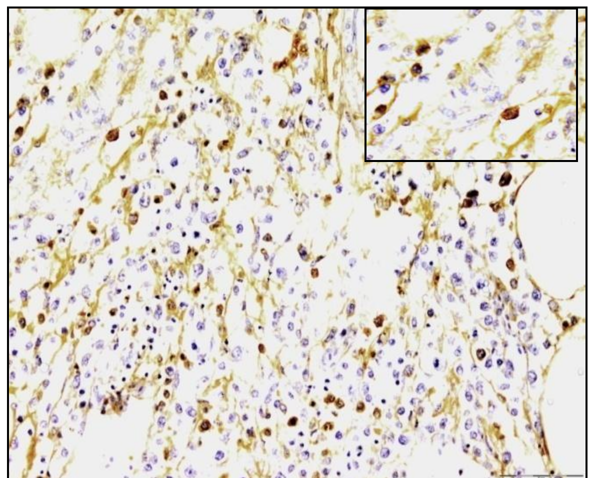

Fig 34: Mesentry showing positive reactivity for MDV antigen.Vectastain ABC staining, counterstained by Gill's haematoxylin x 40X. Inset showing the reactivity more clearly.

Immunohistochemical detection of avian oncogenic viruses in formalin-fixed paraffin embedded tissue sections

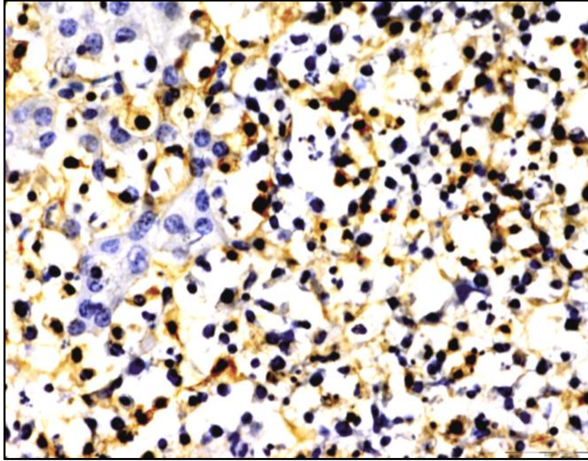

Fig 35: Liver showing positive reactivity for REV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 100X.

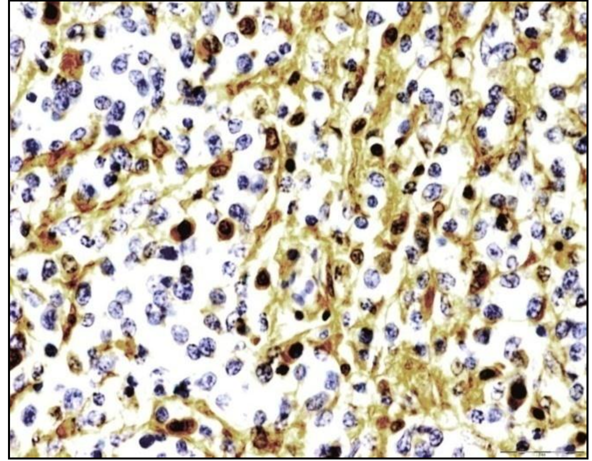

Fig 36: Spleen showing positive reactivity for REV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 100X.

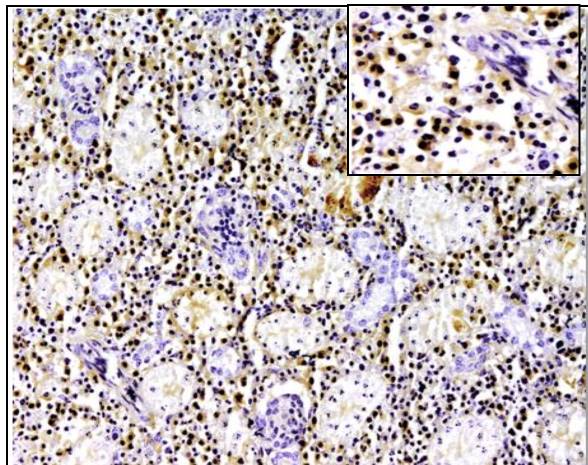

Fig 37: Kidney showing positive reactivity for REV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 40 X. Inset showing the reactivity more clearly.

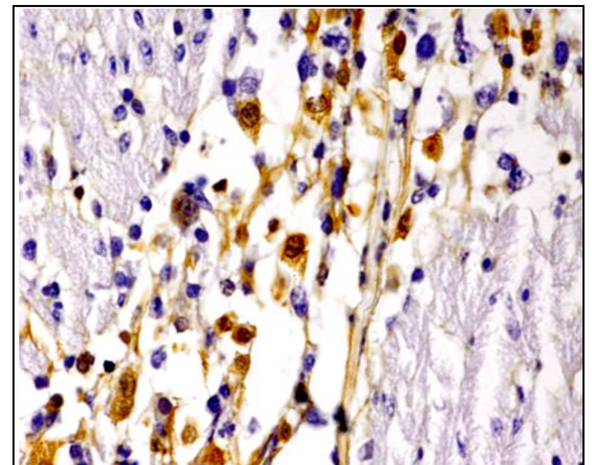

Fig 38: Myocardium showing positive reactivity for REV antigen. Vectastain ABC staining, counterstained by Gill's haematoxylin x 100 X.

Immunohistochemical detection of avian oncogenic viruses in formalin-fixed paraffin embedded tissue sections

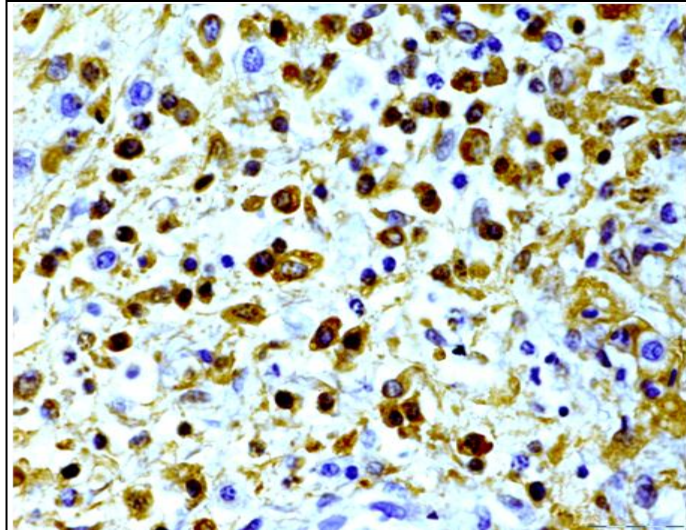

Fig 39: Lungs showing positive reactivity for REV antigen. Vectastain ABC staining, counter-stained by Gill's haematoxylin x 100 X.

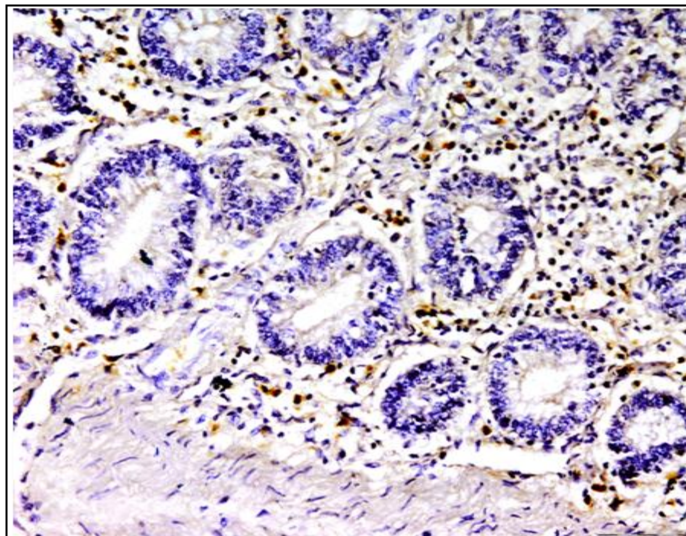

Fig 40: Intestine showing positive reactivity for REV antigen. Vectastain ABC staining, counter-stained by Gill's Haematoxylin x 40 X.

Molecular detection of MDV by Conventional PCR

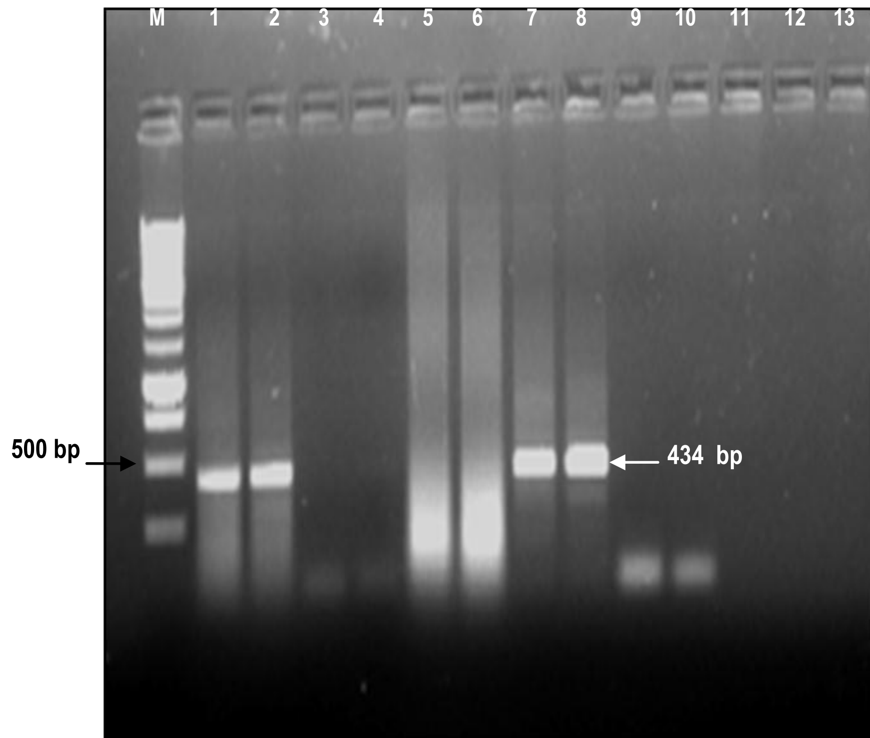

Fig 41: PCR amplified product of MDV

Lane M **1Kb Ladder**
Lane 1 **Sample no.6**
Lane 2 **Sample no.12**
Lane 3 **Sample no.20**
Lane 4 **Sample no.14**
Lane 5 **Sample no.3**
Lane 6 **Sample no.33**
Lane 7 **Sample no.10**
Lane 8 **Sample no.16**
Lane 9 **Negative control**
Lane 10 **Negative control**

Molecular detection of RED by Conventional PCR

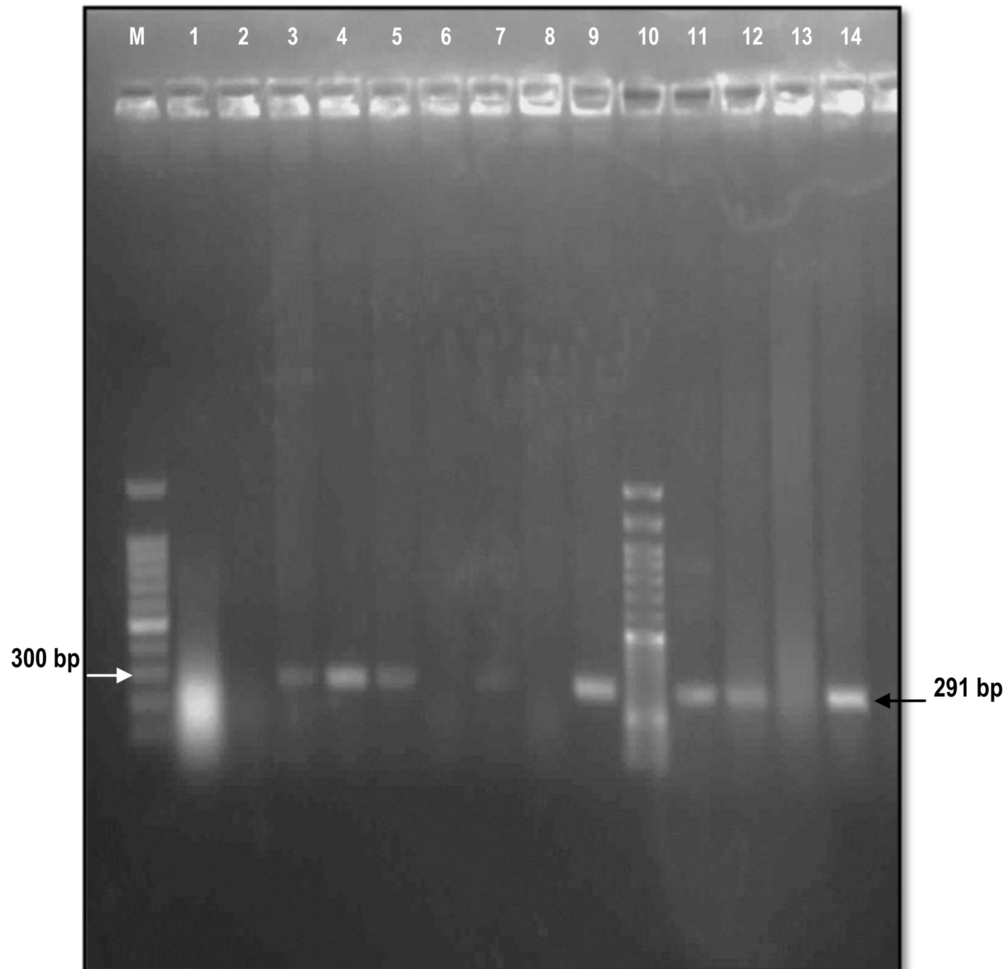

Fig 42: PCR amplified product of REV

Lane M	100 bp Ladder
Lane 1	Sample no.4
Lane 2	Sample no.3
Lane 3	Sample no. 5
Lane 4	Sample no.8
Lane 5	Sample no.9
Lane 6	Sample no.31
Lane 7	Sample no.11
Lane 8	Sample no.16
Lane 9	Sample no. 12
Lane 10	50 bp Ladder
Lane 11	Sample no.17
Lane 12	Sample no.22
Lane 13	Sample no.37
Lane 14	Sample no.24

Detection of multiple avian oncogenic viruses by Multiplex PCR

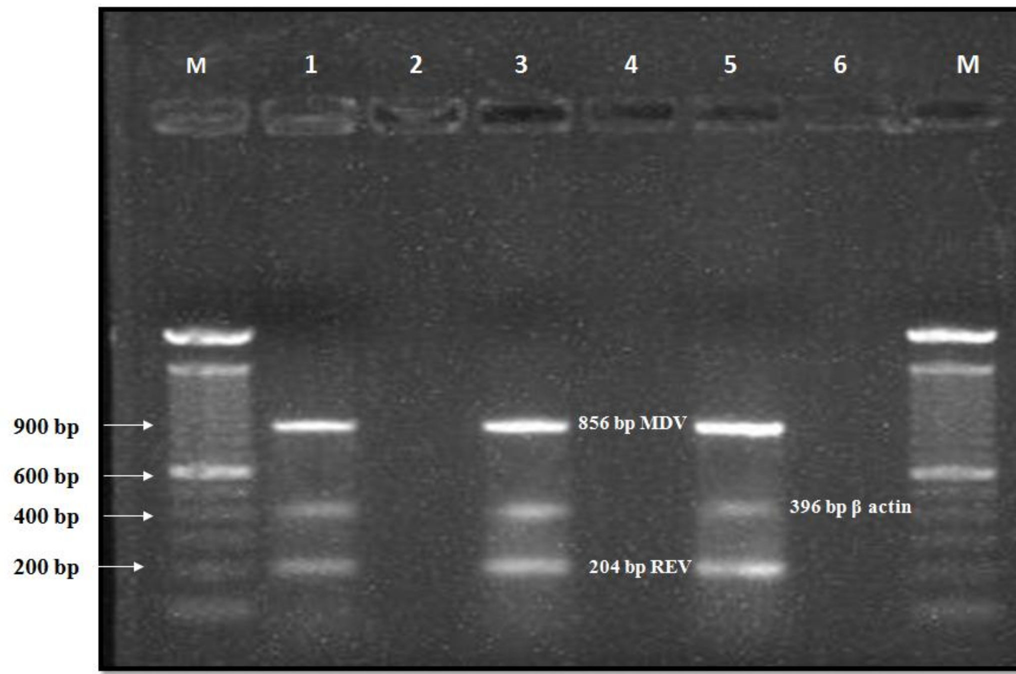

Fig 43: Multiplex PCR amplified product

Lane M	**100bp Ladder**
Lane 1	**Sample no.2**
Lane 2	**Sample no.14**
Lane 3	**Sample no.5**
Lane 4	**Sample no.33**
Lane 5	**Sample no.6**
Lane 6	**Sample no.34**
Lane M	**100bp Ladder**

Detection of multiple avian oncogenic viruses by Multiplex PCR

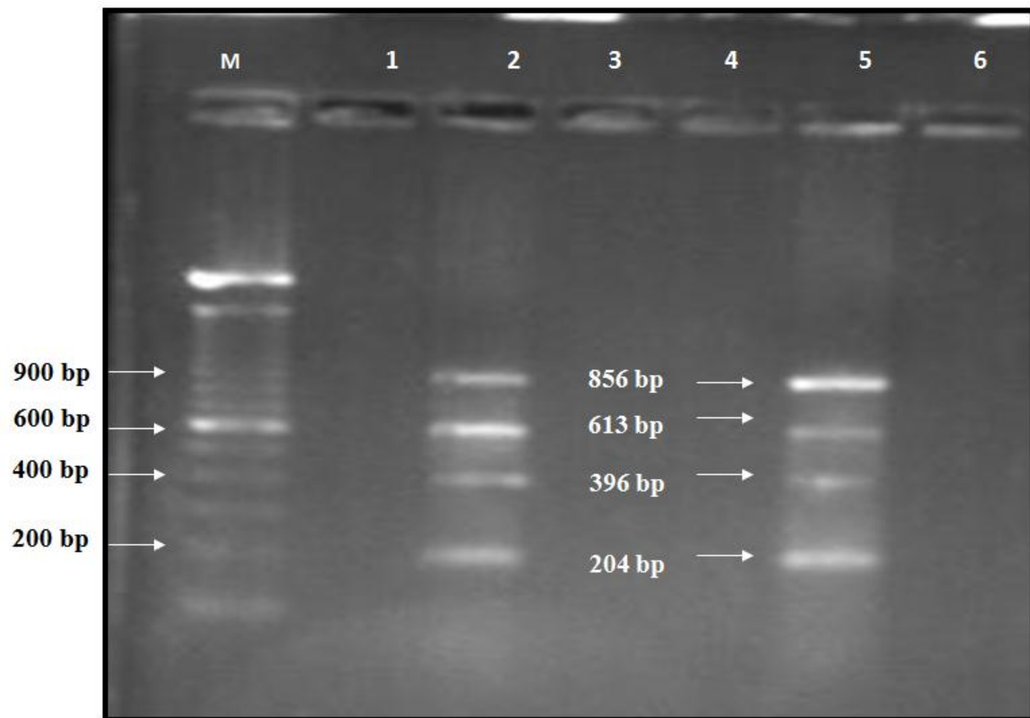

Fig 44: Multiplex PCR amplified product

Lane M	**100bp Ladder**
Lane 1	**Sample no. 13**
Lane 2	**Sample no. 8**
Lane 3	**Sample no.3**
Lane 4	**Sample no. 26**
Lane 5	**Sample no. 21**
Lane 6	**Sample no.36**

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