INCIDENCE OF TUMOURS IN POULTRY WITH SPECIAL REFERENCE TO MAREK'S DISEASE AND LYMPHOID LEUCOSIS

By

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B.V.Sc. & A.H.

**THESIS SUBMITTED TO THE
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CERTIFICATE

Ms. **SWATHI. B** has satisfactorily prosecuted the course of research and that the thesis entitled "**INCIDENCE OF TUMOURS IN POULTRY WITH SPECIAL REFERENCE TO MAREK'S DISEASE AND LYMPHOID LEUCOSIS**" submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part there of has not been previously submitted by her for a degree of any University.

Date: 7/10/12

Place: Hyderabad

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Chairman of the Advisory committee

CERTIFICATE

This is to certify that the thesis entitled **"INCIDENCE OF TUMOURS IN POULTRY WITH SPECIAL REFERENCE TO MAREK'S DISEASE AND LYMPHOID LEUCOSIS"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** in **Veterinary Pathology** of **Sri Venkateswara Veterinary University, Tirupathi** is a record of the bonafide research work carried out by **Dr.B.Swathi** under my guidance and supervision. The student's Advisory committee has approved the subject of the thesis.

No part of the thesis has been submitted for any other degree or diploma. The author of the thesis has duly acknowledged all the assistance and help received during the course of investigation.

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I, **SWATHI. B,** here by declare that the thesis entitled **"INCIDENCE OF TUMOURS IN POULTRY WITH SPECIAL REEFERNCE TO MAREK'S DISEASE AND LYMPHOID LEUCOSIS"** submitted to **Sri Venkateswara Veterinary University** for the Degree of **MASTER OF VETERINARY SCIENCE,** is a result of original research work done by me. It is further declared that the thesis or any part there of has not been published earlier in any manner.

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Swathi Bora

LIST OF ABBREVIATIONS

%	:	Per cent
=	:	More than and equal to
µm	:	Micro meters
µg	:	Micro Gram
µl	:	Micro Liter
^{0}C	:	Degree Celsius
ALV	:	Avian Leucosis Virus
bp	:	Base pairs
DNA	:	Deoxyribo Nucleic Acid
dNTPs	:	Deoxyribo Nucleoside Triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
et al.	:	et alii
fc	:	Final concentration
Fig	:	Figure
FPL	:	Feather Pulp Lesions
g	:	Gram
IU	:	International Unit
kb	:	Kilo base pairs
LL	:	Lymphoid leucosis
M	:	Molar
MD	:	Marek's disease
MDV	:	Marek' Disease Virus

mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
min	:	Minute
ml	:	Milliliter
mM	:	Millimole
PBS	:	Phosphate Buffered Solution
PCNA	:	Proliferating Cell Nuclear Antigen
PCR	:	Polymerase Chain Reaction
pMol	:	Pico mole
ppm	:	Parts per million
RIR	:	Rhode Island Red
rpm	:	Rotations per minute
TBE	:	Tris borate EDTA buffer
vvMDV	:	very virulent Marek's disease virus
WLH	:	White Leg Horn

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ABSTRACT

The aim of the present study was to investigate the incidence of tumours in poultry and diagnose the type of tumor by histopathological and molecular methods. For this purpose, a total of 189 samples from 72 cases were collected and were subjected to various diagnostic methods including cytological, molecular, histopathological and immunohistochemical studies.

The incidence of tumours was recorded as highest (46.93%) in birds ranging from 8 to 18 weeks of age and it was least (4%) in birds of age group less than 8 weeks. The highest incidence of tumours was recorded in Rajasree breed (46%) followed by Rajasree crossbreeds (18%), White Leghorns (12%), Aseel (10%), White Leghorn crossbreeds (6%), Vanaraja (6%) and least in Kadaknath breed (2%). The incidence of tumours was high in female birds (87.5%) when compared with males (12.5%). On the basis of type of tumours, the highest incidence was recorded for

Marek's disease (43.05%) followed by lymphoid leucosis (27.77%), other leukotic tumours (8.33%), hepatoma (4.16%), adenoma (4.16%), fibroma (2.77%), haemangioma (2.77%) and least for granulosa cell tumour, nephroblastoma, osteofibroma, endothelioma and chondroma (1.38%).

Ninty impression smears of various organs or tissues collected from 46 birds was stained with Leishman's / Giemsa stain. Cytological studies revealed that 67.78% of the smears were diagnosed as Marek's disease, and 23.33% of the smears as lymphoid leucosis.

Fifty nine tissue samples suspected for MD and LL were subjected for PCR and 50.85% of the samples were positive for MD, produced an amplicon size of 302bp with primers M1 and M2. 42.37% of the samples were positive for ALV by using pair of primers (AD1 and H5) and produced an amplicon of size 326bp. DNA of 6.78% samples were neither amplified for MDV nor for ALV with any of the respective primers.

Grossly, 25%of 72 cases were suspected for Marek's disease, 62.5% for Lymphoid leucosis, 9.72% for fibromas and 2.77% for haemangiomas.

A total of 189 tissue samples from 72 cases were subjected to histopathological studies and 120 (63.49%) samples were confirmed as Marek's disease, 55 (29.10%) as LL and 14 (7.4%) as other neoplastic conditions which included cases of adenoma, hepatoma, haemangioma, fibroma, nephroblastoma, chondroma, granulosa cell tumour, endothelioma and osteofibroma.

A total of 29 samples were subjected to immunohistochemical staining technique for PCNA and observed PCNA labelling in the tumour tissue and demonstrated different levels of staining intensity and indicated the activity of the cells.

INTRODUCTION

CHAPTER 1

Introduction

Of all the avian neoplastic conditions, Marek's disease and Avian Leucosis Complex were given much importance due to the significant economic losses due to lowered production and mortality caused by them and their frequency of occurrence.

Tumours in domestic fowl were first described by Sedamgrotski (1876, cited by Jackson, 1936) and the most comprehensive descriptive review in this field was brought out by Joest and Ernesti (1916). Tumours in poultry are either viral or of unknown origin. Most of the neoplastic conditions are due to viruses that include Marek's disease virus (MDV), Avian Leucosis Virus (ALV), Reticuloendotheliosis Virus (REV) and Lymphoproliferative Disease Virus (LPDV). These viruses are immunosuppressive in addition to oncogenic property (Davidson and Borenshtain, 1999).

Incidence of neoplasms other than lymphoid tumours appears to be low. Virus induced tumours are principally of mesodermal in origin and are transmissible. Marek's disease virus (MDV), serotype I Herpes virus, transforms T lymphocytes and causes tumours of peripheral nerves, skin, visceral and other organs. Reticuloendotheliosis virus (REV), Avian Leucosis Virus (ALV) and Lymphoproliferative Disease Virus (LPDV) are avian C type Retroviruses. Reticuloendotheliosis virus transforms pre-B and pre-T lymphocytes causing bursal and T cell lymphomas in susceptible chickens and turkeys which often closely resemble MDV lymphomas. ALVs represent a distinct type of simple

alpha retroviruses that are classified into 10 subgroups (A to J) according to the properties of viral envelope glycoprotein underlying their immunological cross reactivity, viral cross interference and host range (Hunter,1997). ALV (A-E) transforms B lymphocytes causing bursal lymphomas in chicken. It also causes other tumours like sarcomas and other connective tissue tumours. ALV-J transforms myeloid cells causing myelocytomatosis, predominantly in bones mainly of meat type broilers. Lymphoproliferative disease virus affects turkeys sporadically and the target cell for transformation is not known (Davidson and Silva, 2008).

In recent years meager work was carried out on the incidence of neoplasms in poultry in India as well as in Andhra Pradesh state but much emphasis has been made on the diagnosis of tumours since control measures against avian neoplasms may vary according to the etiology. Keeping in view of the importance of neoplasms and their diagnosis, the present study is intended with the following objectives:

1) To know the incidence of tumours in poultry

2) To diagnose the type of tumour by histopathological and molecular methods.

REVIEW OF LITERATURE

CHAPTER 2

Review of Literature

Neoplasms in poultry are generally recognised as one of the more common disease conditions of the domestic chicken. Various factors will obviously affect the result of any survey on the incidence and of them some are age, genetic composition or inheritable tendencies, and certain other factors as yet unknown which may result in high incidence. Much of work has been carried out on the incidence of tumours in poultry in India and in Andhra Pradesh as well, during last few decades but during recent past decades the reports / literature available were scanty. Keeping in view of the above, the study on incidence of tumours in poultry was taken up and the related available literature is reviewed.

2.1 INCIDENCE OF TUMOURS

2.1.1 In India

Iyer (1960) reported 72 (26.1%) tumour cases among 276 avian specimens examined as avian lymphocytoma and leucosis (56), adenoma and cystic adenoma (5), leiomyoma (4), malignant hepatoma (2), haemangioma (2), adenocarcinoma (2) and one each of granulosa cell tumour and fibrosarcoma. The gross lesions of fibrosarcoma in one year old bird showed enlarged liver with greyish White irregular nodular masses and histologically, the tumour mass consisted of spindle shape cells with numerous mitotic figures supported by a stroma of delicate blood vessels.

Connective tissue in the tumour was demonstrated by van Gieson and Haidenheins "azan" variant.

Ovarian granulosa cell tumour in a hen was first reported in India by Shukla and Iyer (1960). They described that it was lobulated, oval shaped, yellowish White in colour, encapsulated, well pedunculated and of walnut size observed on the left ovary and histologically presence of mature granulosa cells, interspersed with delicate blood vessels and filamentous connective strands, confirmed by van Gieson's method of staining replacing the normal parenchyma of the ovary.

Unique cases of cavernous haemangioma in RIR chicken liver (Christopher and Rao, 1964) and leiomyoma in pigeon liver (Christopher *et al.*, 1965) were reported. Haemangioma grossly revealed reddish specks on enlarged liver and histologically numerous dilated spaces of varying size and shape, lined by thin endothelial cells with scanty cytoplasm and moderately large nucleus, distributed over many lobules in the liver.

Singh and Singh (1968) reported two cases of fibromas located just above the rim of the eyes among 9000 birds examined. Grossly, the tumours were large, solitary masses which were firm, encapsulated and revealed slight lobulations. Microscopically, there were predominant and varying numbers of fibroblast cells, having spindle shaped nuclei and the connective tissue was demonstrated by Mallory's triple stain.

Sharma (1968) reported a case of an angiosarcoma in the ovary and a malignant hepatoma in poultry.

Papillary adenoma attached to bronchial mucosa was described grossly as soft, yellowish White cauliflower like growth and histologically consisted of delicate stroma of vascular connective tissue in which numerous glandular structures were

present, the epithelium of which was thrown into numerous papillary masses and histiocytic sarcoma located at the base of neck was a firm lemon sized growth, pale and encapsulated and histologically consisted of spindle shaped fibroblasts arranged in bundles or in whorls associated with varying quantities of collagen fibrils and polygonal cells of the histiocytic type (Rao *et al.*,1967). Sastry *et al.* (1967) reported 3 cases of haemangiopericytoma, 2 in fowl and 1 in a bullock, in Andhra Pradesh.

Sharma and Singh (1968), in their studies on pathology of female genital tract of poultry with special reference to egg peritonitis, observed that 20 (7.8%) birds were suffering with neoplasm in the female genital organs among the 255 hens. The types of neoplasms recorded were lymphocytomatosis (11), granulosa cell tumour (7), fibroleimyoma (1) and leiomyoma (1).

Kolte *et al.* (1968) reported a case of hepatocellular carcinoma in a 4 month old aged White Leghorn pullet. Grossly, liver was enlarged with scattering yellowish White growths. Histologically, cellular pattern appeared to be alveolar or tubular and rarely papillary with scanty connective tissue stroma made up of endothelial cells. The nuclei appeared slightly hyperchromatic with prominent nucleolus with few mitotic figures.

Awadhiya *et al.* (1968) reported 9 cases of smooth muscle tumours in the fowls of which 8 were leiomyoma and one leiomyosarcoma. The growths were of variable shapes like roughly globular, heart shaped, elliptical etc., and the colour was pink. Microscopically, tumour was composed of interlacing bundles of smooth muscle fibres cut in different planes, scanty fibrous tissue stroma and a few atypical mitotic figures.

adenocarcinomas involving the ovaries (1), oviducts (9), metastatic growths in the mesentery (14), ovarian thecoma (1), oviduct carcinoma (1), carcinoma leimyomatosum in mesentery and oviduct (2), heart fibrosarcoma (1), histiocytic cell sarcoma in the striated muscle (1), liver haemangioma (2) and liver cholangioma (1) were the other neoplasms recorded.

Sahota and Singh (1977) reported 477 tumours in ovary and/or oviduct of 16,214 adult birds examined. Marek's disease (17 ovaries and 1 oviduct), lymphoid leucosis (21 ovaries and 17 oviducts), adenocarcinomas (56 ovaries and 66 oviducts), granulosa cell tumour (5 ovaries), theca cell tumour (1 ovary), adenoma of oviduct (5), leiomyoma of the oviduct and mesosalpinx (5), leiomyoma of mesosalpinx (235), leiomyoma-haemangiomatosum of mesosalpinx (9), mixed cell tumour (9) and concomitant tumours (21) were recorded.

Deka and Grewal (1982) reported 267 cases of tumourous growths and recorded the incidence of neoplasms to be 0.59%. They observed higher incidence of neoplasms in White Leghorn adult hens in comparison with Indian Desi fowl and further they opined that the incidence was high in the month of March. They observed that kidneys were more commonly affected than other visceral organs and the incidence of malignant tumours was higher than benign.

A total of 1798 neoplastic growths were examined during a 12 year period by Choudary and Rao (1986). Grossly a case of thecoma was spherical with smooth glistening surface located on ovary of a hen and histologically, the growth consisted of spindle cells in bundles with vesicular nuclei and fat vacuoles in the cytoplasm.

Bhoomaiah *et al.* (1987) reported 15 cases (0.72%) of adenocarcinomas of intestines among 6950 birds examined.

Brar and Grewal (1989) observed eight (0.03%) cases of non leukotic tumours affecting skin, among 25000 birds examined. They recorded cases of haemangioma, fibroma, papilloma and myxoma involving skin of domestic fowl.

A case of cystic adenocarcinoma of ovary was reported in a Khaki Campbell duck. Grossly, it was a large growth originating from ovary, consisted of approximately 320 cysts of varying sizes. Other organs were atrophied except liver as it showed enlargement with diffuse greyish White raised foci of variable sizes and microscopically both organs consisted of multiple small and large cysts lined by one or more layers of the cuboidal epithelial cells. Tanwani *et al.* (1991) opined that trans-coelomic transplantation of metastasis in adenocarcinomas of ovary. Another case of ovarian adenocarcinoma was reported in a guinea fowl by Kumar *et al.* (2004).

2.1.2 In Abroad

Tyzzer and Ordway (1909) reported nine cases of tumours in seven birds out of 4000 birds examined and described as myxosarcoma (1), lymphoma (7) and leiomyoma of the mesentery (1).

Goss (1940) recorded tumours in 19.5% birds out of 7408 birds examined. Olson (1942) observed occurrence of tumours in small poultry flock of 48 and reported that 13 birds (27%) had died with neoplastic diseases. In another study, Olson and Bullis (1942) reported the incidence of tumours to be 12.9% among 2304 birds examined over a period of 2 years.

Campbell (1945) investigated neoplastic disease of fowl with special reference to its history, incidence and seasonal variation. He reported 386 (18.7%) cases of neoplasia among 2063 chickens examined over a period of 5 years and reported that neoplasms were most common in the Buff Rock breed (38.8%) and least

in White Wyandottes (10.3%) and indicated that there was probably a significant seasonal variation of neoplastic disease in fowls.

Jarplid (1961) conducted a systematic study on incidence of tumours in poultry over a period of 6 years and reported that 27 out of 433 tumours (leucosis and neurolymphomatosis not included) recorded were of vascular origin and all the tumours were haemangio endotheliomas.

Feldman and Olson (1965) cited reports of various workers who had worked on avian neoplasms between 1915 and 1955 and indicated an incidence of neoplasms (other than leucosis) from 3 to 19%.

Spontaneously occurring tumours in the duck namely carcinoma in a 2 year old female White Pekin, a teratoma in 2 years old male White Pekin and a case of fibrosarcoma in 2 year old female White Pekin were reported by Rigdon (1970).

During an investigation period of 10 years in Iran, a total of 16016 domestic fowls were examined, of which 64 (0.4%) were affected with neoplastic disease other than leucosis complex. The most frequently occurring neoplasms were leiomyoma, adenocarcinoma and fibroma (Naghshineh and Afnan, 1974).

Bergmann *et al.* (1984) observed occurrence of neoplastic disease in slaughter hens and reported that neoplasms were the third most important cause of rejection in slaughter hens, accounting for 10% of all condemnations and 0.7% of the number slaughtered. The commonest neoplasm recorded was adenocarcinomatosis (71.71%), followed by leucosis (8.52%), nephroblastoma (5.68%), Marek's disease (5.62%), leiomyoma (3.26%) and sarcomatoses (1.09%).

Naturally occurring neoplasms were observed in avian species by Reece (1992) over a period of 10 years and reported 383 cases of tumours out of (=) 10,000

Berry *et al.* (2006) examined hens at 5 years of age for the presence of oviduct-associated fibroid tumours and they found tumours attached to the internal surface of the oviduct, embedded in the oviduct wall, or attached to the exterior of the magnum and isthmus. Tumour samples were subjected for histological analyses or immunohistochemistry for estrogen and progesterone receptors, proliferating cell nuclear antigen (PCNA) and Bcl-2 protein expression and found positive for those receptors. Immunohistochemical examinations of hen fibroid and adjacent normal oviduct tissue demonstrated PCNA labelling in fibroid tissue, with greater staining intensity at the interface of the normal oviduct and fibroid tissue and the outer edges of the fibroid body.

Ozyigit and Sonmez (2007) demonstrated the presence of estrogen and progesterone receptors (ERs and PRs, respectively) in oviduct adenocarcinomas in laying hens. In all, 32 oviduct adenocarcinomas (18 primary tumours and 14 intestinal metastases) from Babcock B-380 laying hens of 16-18 months old were stained with commercially available monoclonal antibodies against mouse-anti-human ERs and mouse-anti-human PRs and reported that 13 tumours (7 oviduct adenocarcinomas and 6 intestinal metastases) stained positively for ERs and 12 tumours (4 oviduct adenocarcinomas and 8 intestinal metastases) gave a positive reaction to PR antibody.

2.3 INCIDENCE AND DIAGNOSIS OF MAREK'S DISEASE

Sharma *et al.* (1972) reported four spontaneous cases of MD in naturally as well as experimentally infected birds. Grossly, the feather follicles were enlarged and skin surface had varying sized tumourous nodules and histopathologically, patchy infiltration of skin with pleomorphic and lymphoid cells, and presence of intranuclear eosinophilic inclusion bodies in the feather follicles were observed.

Panda *et al.* (1983) observed higher mortality (28.15%) due to MD at 21 to 40 weeks of age as compared to 8.86% mortality at 9 to 20 weeks of age. The post mortem findings revealed tumours in liver, spleen, pancreas, kidney, heart, ovary, muscles and skin. Nerve lesions were mainly recorded in brachial and sciatic plexuses and the mortality rate was found higher in females than in males.

Dave (1989) studied the outbreaks of acute form of MD in five successive flocks of WLH birds at Poultry Complex of Gujarat Agricultural University, Anand. Mortality due to acute MD ranged from 12.30 to 30.09%.

Cho *et al.* (1998) reported the use of cytology of feather pulp lesions (FPL) for diagnosis and prediction of MD. They found that birds having MD lymphoma or nerve lesions exhibited the characteristic changes on the cytological smears of FPL, comprising initial non-suppurative inflammatory to the late lymphomatous FPL.

Sung (2002) found that the incidence of MD suddenly increased in 1997 in Korea. Most MD cases in this country were detected in chicken over 20 wk of age. Five MDV isolate were obtained from field flocks, in which severe losses had occurred, and one of the virus isolate was studied to compare its pathotype with the prototype JM strain. The isolate KOMD-IC induced severe depression not only in body weight but also in relative bursal weight, and the depression by KOMD-IC was more severe than that induced by JM strain. In addition, the incidence of MD tumour caused by KOMD-IC was higher than that caused by the JM strain.

Balachandran *et al.* (2009) conducted a histopathological survey of Marek's disease and lymphoid leucosis in chicken tissue samples collected from broilers as well as layer birds and reported the incidence of MD as 6.85% in broilers and 13.54% in layers and the incidence of ALV as 2.16% of layer birds. Out of 767 tissues collected five tissues (6.85%) from broilers and 94 tissues (13.54%) from

layers had lesions suggestive of Marek's disease. Marek's disease involved the liver (34.34%), spleen (26.26%), kidneys (12.12%), ovaries (7.07%), proventriculus (8.08%), lung (4.04%), sciatic nerve (3.03%), intestine (2.02%), skin (1.01%) and mesentery (1.01%).

2.3.1 Molecular diagnosis of MDV

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 dimer or a trimer 132 bp repeats in pathogenic MDV-1 DNA from blood and organs of commercial chicken with symptoms of MD.

Silva (1992) differentiated pathogenic and non-pathogenic serotype-1 MDVs by PCR amplification of the 132 bp tandem direct repeats within the MDV genome. The PCR reaction was specific for serotype-1 MDVs, amplifying fragments corresponding to one to three copies of the tandem repeats present in Md11/8, JM/102W and GA viruses. A high molecular weight DNA smear was observed when the DNA from an attenuated MDV (Md11/100) was amplified. The technique allowed the detection of two copies of the 132 bp repeats in the DNA extracted from MDV induced lymphomas removed from two chickens. No DNA was amplified from the DNA extracted from lymphomas induced by either avian leucosis virus (RAV-1) or reticuloendotheliosis virus (chick syncytial virus).

In a similar study, Zhu *et al.* (1992) reported the differentiation of oncogenic and non-oncogenic strains of MDV type 1 by PCR, using primers chosen from the sequences within the long inverted repeats of MDV1 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 MDV1 and HVT were also used for determination of their serotype specificity. The PCR procedure was found to be simple and sensitive for identification of MDV1 and HVT, as well as for estimation of oncogenicity of MDV1.

Becker *et al.* (1993) developed a radioactive PCR test that amplified vvMDV- 1 DNA sequence containing the 132 bp repeats. In apathogenic MDV-1 (CVI 988, Rispense), amplified DNA band containing multiple copies of 132 bp repeats were identified. PCR was also used to monitor the passage level of vvMDV-1 in chicken embryo fibroblast (CEF) cell culture, in which the number of tandem 132 bp repeats was found increasing. At passage level 32 of vvMDV-1 B isolate, the 132 bp tandem repeat was markedly amplified and its pattern resembled that of the MDV-1 (CVI 988, Rispense) vaccine virus. The PCR test demonstrated that a pathogenic MDV-1 Md11/75c virus passaged extensively *in vitro* had amplified 132 bp DNA repeats similar to those of the commercial vaccine viruses (CVI 988, Rispense).

Davidson *et al.* (1995) reported the use of PCR for the diagnosis of natural infection of chicken and turkeys with MDV and reticuloendotheliosis virus.

Bumstead *et al.* (1997) described quantification of MDV in chicken lymphocytes using the PCR with fluorescence detection. The primers were tagged with fluorescent. The assay determines the numbers of viral genomes present in samples by PCR amplification of a portion of the viral genome for a restricted number of cycles. Fluorescent-tagged primers were used for the PCR amplification, which allowed quantification of the fluorescent product.

Sung *et al.* (1997) carried out a survey of MD in a broiler farm using PCR to detect pathogenic MDV. PCR was carried out using the primers chosen from

sequences that flank the 132 bp tandem direct repeats. The size of major amplified products for DNAs of JM10 and 88-57 (a Korean isolate) was 460 bp, indicating that these viral DNA contained mainly two copies of 132 bp repeats. However, the amplified product of the DNA extracted from attenuated serotype-1 MDV (CVI 988 Rispense) was variable in size.

Borenshtein and Davidson (1999) reported the development of a two-step PCR, the Hot Spot-combined PCR assay for the identification and characterization of recombinant viruses in Marek's disease (herpes) and retrovirus co-infections.

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. They found that a more virulent strain of MDV (648 A) replicated well in thymus during cytolytic infection than did a less virulent strain.

Handberg *et al.* (2001) developed a method to detect serotype-1 and serotype- 3 MDV by specific PCR. The method was applied to various tissue samples from chicken experimentally inoculated with serotype-1 or serotype-3 MDV. The serotype-1 strains of CVI 988 and RB-1B could be detected in feather follicle epithelium up to 56 and 84 days post-inoculation (PI.) respectively, while the MDV-3 serotype was detected until 42 days PI. In addition the PCR was applied to the samples collected from four commercial table egg layer flocks of young stock or pullets vaccinated with either serotype-1 or serotype-3 (HVT) vaccine, which had various clinical signs of MD. MDV-1 was detected in buffy-coat cells, spleen, liver, skin, feather tips and ovaries. The detection of MDV in feather tips appeared to be as sensitive as co cultivation of buffy coat cells.

Islam *et al.* (2001) investigated the factors that might influence the interpretation of PCR results in commercial broiler chickens including the effect of route of infection and HVT vaccination status. They also investigated the suitability of PBL and spleen as tissue samples for MDV detection. Over all data revealed that route of infection (intraperitoneal) verses inhalation had minor effects on the timings of subsequent MDV detection by PCR.

Davidson and Borenshtein (2002) aimed at examining the efficacy of amplifying MDV and / or ALV-J feather tip DNA as compared to DNA purified from liver and spleen. They found that the PCR for MDV and ALV-5 using DNA from feather tips was more effective for diagnosis of naturally infected commercial chicken than using the liver and spleen.

Levy *et al.* (2003) quantified MDV DNA in a cultured cell by duplex real time PCR using the host ovotransterin gene as an internal standard. They used a plasmid containing MDV IC P4 gene sequence as a standard and fluorescent PCR product were electrophoresed and quantified using the Applied Biosystems Genescan Software.

Islam *et al.* (2004) developed Quantitative real-time PCR (qPCR) assays for the three serotypes of MDV. An internal control qPCR assay that detected chicken a2 (VI) collagen gene was also developed to allow quantitation of MDV. To reduce cost and time, the assays for MDV1 and the internal control were combined into a duplex assay. The MDV qPCR assays were specific to their target gene when compared using Australian field and vaccine strains of MDV and 10 to 100 fold more sensitive than standard PCR.

Gimeno *et al.* (2005) reported a novel criterion for diagnosis of MDV induced lymphoma after examining the several criteria to assist in the differential

diagnosis of tumours induced by MDV from those induced by avian leucosis virus (ALV) and reticuloendotheliosis virus (REV). A collection of tumours induced by inoculation of specific strains of MDV, ALV and REV, alone or in combination, were tested for quantification of MDV DNA by real-time polymerase chain reaction, expression of the MDV oncogene Meq, expression of several cell markers associated with transformation (CD30, Marek's disease-associated surface antigen, and p53), and level of DNA methylation in the tumour cells. In addition, tissues latently infected with MDV and non-infected tissues were tested as controls. Tumours induced by MDV had about 10(2)-fold more copies of MDV DNA than either tissues latently infected by MDV or tumours induced by retrovirus in MDV-vaccinated chickens. Moreover, the MDV antigen Meq was consistently expressed in all MDV tumours, but it could not be detected in tissues latently infected with MDV or in tumours induced by retrovirus in MDV-vaccinated chickens. Other markers studied were not specific for MDV and therefore reported that they had limited value for diagnosis.

Kalyani (2006) reported an overall mortality rate due to MD in two major poultry zones of Gujarat viz. Anand and Mahuva was 3.30 with highest mortality (7.3 %) in a commercial farm in Anand Zone on the basis of clinical signs and post mortem lesions and by detection of different serotypes of MDV from clinical cases exhibiting frank lesions of MD by PCR technique using six pairs of primers.

Murata *et al.* (2007) Developed a nested polymerase chain reaction method to detect oncogenic Marek's disease virus from feather tips of infected birds and they opined that the method is appropriate for the surveillance of the highly virulent MDV infection in the field.

Raja *et al.* (2009) monitored for the MDV virus occurrence in the field and they isolated genome from feather follicles, spleen, and liver of the chicken (173

samples) of which twenty two samples (12.7%) were positive for MDV genome in PCR and belonged to the serotype 1. In situ hybridization studies also confirmed a presence of MDV serotype 1 in the infected liver tissues and showed the presence of virulent/very virulent MDV strains in the Indian poultry flocks.

2.4 INCIDENCE AND DIAGNOSIS OF AVIAN LEUCOSIS

The incidence of Avian Leucosis was long back recognized by Roloff (1869). Viraraghavan *et al.* (1965) reported 132 cases (13.9%) of Avian Leucosis Complex (ALC) among 952 chickens examined with high incidence in White Leghorns and equal proportions among males and females. They opined that season played a significant role in the incidence of ALC. Distribution of lesions in different organs and tissues was recorded and concluded the order of frequency as liver, kidney, spleen and ovary.

Soman and Sethi (1972) conducted study on incidence of different forms of Avian Leucosis Complex (ALC) as influenced by breed, age, sex and season. They reported 64 cases of different forms of ALC confirmed by cytological and histopathological studies. Forty one cases of lymphoid leucosis, 6 erythroleucosis, 14 occular lymphomatosis and 3 fibrosarcomas were recorded among 64 cases. ALC accounted for 16.2% of mortality among 339 birds examined. It was observed that White Leghorns are more susceptible than Rhode Island Red; females were more susceptible than males and an insignificant influence of season on incidence.

Damodaran and Thanikachalam (1973) reported that the incidence of Avian Leucosis Complex in Madras was 2.67%.

In the epidemiological studies conducted by Zelenskii *et al.* (1980) leucosis was diagnosed in 5 to 25% of birds up to four years during a survey of the breeding

and commercial poultry industry, the highest mortality rates in the first two years of life. Leucosis was diagnosed in 0.05 to 7% of birds above four years of age.

Wadsworth (1981) reported 6 cases of lymphoid leucosis (0.4%) among 1234 post mortem examination of captive wild birds during a 5 years observation period at laboratory of Zoological society.

Rao and Choudary (1981) reported that 826 out of 1393 neoplasms observed were the cases of lymphoid leucosis (59%). Other tumours recorded were Marek's disease (551), fibroma (5), leiomyoma (4), haemangioma (2) and one each of granulosa cell tumour, hepatoma, adenocarcinoma, fibrosarcoma and chondroma.

Reddy (1990) observed 9 cases each of lymphoid leucosis in ovaries cases and 9 oviducts (0.45%) out of 1980 hens necropsied.

Ghosh *et al.* (2004) conducted pathological studies in organs of highly productive layer lines infected with subgroup A and C Rous sarcoma virus. Grossly, liver became yellowish brown with a large number of sago sized growths scattered on its surface while in some cases the growth was diffusely scattered. Similar growths were also seen in heart, lungs, spleen and kidneys. Histopathologically, fibroblast proliferation was observed displacing and compressing the native tissue in all organs.

Mayumi *et al.* (2005) examined 91 cases of tumours of various organs on chicken histopathologically and gave the conclusion that 70 of 91 cases were AL (include 60 cases LL, 10 of ML), and 21 were of other diseases. They opined that histopathological examination was useful in diagnosis of chicken Avian Leucosis.

2.4.1 Molecular diagnosis of ALV

Smith *et al.* (1998a) developed a PCR assay for the detection of avian leucosis virus strain J (ALV-J) in chickens. Primers were based on the element and

the 3' terminus of the long terminal repeat of proviral ALV-J. PCR products were amplified from genomic DNA infected with either strain HPRS-103, the prototype of ALV-J, or field isolates of ALV-J obtained from broiler breeders flocks that exhibited myeloid leucosis. They concluded that the newly developed PCR offered a specific and sensitive alternative to conventional virus isolation tests for ALV-J.

Latif and Khalafalla (2005) reported first case of ALV incidence in Sudan. PCR tests were carried out by extracting DNA from ALV infected livers and spleen, and confirmed that ALV subgroup (A-D), HPRS and ALV-J were involved in the infection.

Silva *et al.* (2007) designed PCR primers that would specifically detect and amplify viruses from each of the 6 ALV subgroups: A, B, C, D, E and J. Subgroup B and D envelopes are related, and B specific primers also amplified subgroup D viruses. They demonstrated that the contaminant in 2 commercial Marek's disease vaccine was subgroup A ALV.

Acevedo *et al.* (2009) detected the possible presence of ALV DNA as contaminant of Marek's disease vaccines intended to be used in poultry by ALV specific PCR assay. DNA samples extracted from seven vaccines were subjected to PCR using primers of a conserved region of *env* gene of HPRS-103 and ALV sequences were detected in all seven samples (100%). They concluded that PCR was useful for the detection of ALVs as contaminants of imported Marek's disease vaccines.

MATERIALS AND METHODS

CHAPTER 3

Materials and Methods

The birds of various age groups presented to the department for necropsy examination, samples collected from Avian Health division of Project Directorate on Poultry, Hyderabad and from other private farms in and around Hyderabad at necropsy were used as the materials for the present study. The information pertaining to the samples of study were recorded.

All the dead birds were necropsied systematically as per the standard procedure described by Bermudez and Brown (2003). All the organs were examined and any growths on the organs were recorded with reference to location, size, shape, color, consistency etc. and the impression smears of the organs and growths were taken on clean grease free slides for cytological examinations and growth pieces on ice were collected and preserved at -20^{0}C for molecular and immunohistochemical studies. Further pieces of growths were collected in 10% neutral buffered formal saline for histopathological examination.

One hundred and eighty nine samples (suspected growths and organs) were collected from 71 cases to carry out cytological, molecular, histopathological and immunohistochemical studies.

3.1 CYTOLOGY

The collected organ impression smears (90) were stained with Leishman's or Giemsa stain (Viraraghavan and Nair, 1965). Few smears were stained by Gram's stain and Lactophenol cotton blue stain to rule out the presence of other infections.

3.2 MOLECULAR STUDIES

3.2.1 Preparation of DNA:

Tissues (54) stored at -20^{0}C were subjected for DNA extraction (Murata *et al.*, 2007). The tissue was homogenized and incubated at 37^{0}C for 2 hours in 500µl of the lysis buffer (7.5 µl) containing proteinase K (Genei, Bangalore, India) (fc 20 mg/ml). Total cellular DNA was extracted in 500µl of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma, USA) (Handberg *et al.*, 2001). The contents in the tubes were vortexed for thorough mixing of the contents and centrifuged at 14,000 rpm for 15 min. The DNA was precipitated by addition of double the volume of cold absolute isopropanol to the aqueous phase. The tubes were incubated for 15 min at room temperature and centrifuged at 14,000 rpm for 15 min. The pellet was treated with 70% isopropanol and centrifuged at 10,000 rpm for 10 min. The pellet was dried at room temperature and the DNA was collected by diluting the pellet with autoclaved deionized triple distilled water and stored at -20^{0}C for further use.

3.2.2 Polymerase Chain Reaction (PCR):

Oligonucleotide primers:

For MDV, the primers described previously by Becker *et al.* (1992) and Davidson *et al.* (1995) were used. They are selected to detect the 132 bp nucleotide repeat sequence located within the BamH1-H fragment (Bradley *et al.*, 1989) by employing sequences flanking the tandem repeat sequence. The forward primer M1: 5' TACTTCCTATATAGATTGAGACGT 3' (24 mer) and the reverse primer M2: 5' GAGATCCTGGTAAGGTGTAATATA 3' (24 mer) procured from Integrated DNA Technologies, Inc., India were used for detecting MDV. The forward primer was located at 65 bp 5' to the tandem 132 bp repeat and the reverse primer is at 105 bp downstream (Davidson *et al.*, 1995).

The sequence of the oligonucleotide primers used to detect ALV in this study were derived from the published sequences (Bai *et al.*, 1995) and used according to Smith *et al.* (1998b) and Latif and Khalafalla (2005). Sequence of the oligonucleotide primers, their targets, position on genome and expected PCR product size is as given below:

Primer	Sequence 5'-3'	Product size with H5	Position	Amplification targets	Source
H5 F	GGATGAGGTGACTAAGA AAG	295-326 bp with H5	525?-5277	-	Bai *et al.* (1995) and Latif and Khalafalla (2005).
AD1 R	GGGAGGTGGCTGACTGT GT		5327-5345	Subgroups A-E ALV	

Primer H5 was designed that flanked the 3' region of the *pol* gene, which is conserved across several ALV subgroups. Primer AD1 when used with H5 is expected to give 292-326 bp which was conserved among ALV subgroups A, B, C, D and E (Latif and Khalafalla, 2005). All the primers used in this study were got synthesized from Integrated DNA Technologies (IDT), Inc., India.

PCR amplification protocol:

PCR for both MDV and ALV was performed in a final volume of 25 µl containing 10X PCR buffer, $MgCl_2$ (25 mM), dNTPs (10mM) (MBI Fermentas, Hanover, USA), primers (20pM/µl) and Taq polymerase (Genei, Bangalore, India). The composition of the reaction was as follows:

Composition of PCR mixture:

	X 1
10x assay buffer	2.5µl
$MgCl_2$ (25 mM)	1.5µl
dNTPs (10 mM)	0.125µl
Primer (20 pm/µl) (F)	0.5µl
Primer (20 pm/µl) (R)	0.5µl
Taq Polymerase 5U/µl	0.2µl
DNA template	3.0µl
Autoclaved deionized triple distilled water	16.675µl
Total	25 µl

PCR cycling conditions:

Amplification was carried out in thermal cycler (Bioer-Xp Cycler, Imperial Life Science) with the following conditions (Smith *et al.*, 1998b).

Stage of Cycle	Number of cycles	Temperature	Time Duration
Initiation	1	94^{0}C	4 min
Denaturation		94^{0}C	1 min
Annealing	35 each	55^{0}C	1 min
Synthesis		72^{0}C	1 min
Final elongation	1	72^{0}C	4 min
Holding		4^{0}C	

3.2.3 Analysis of PCR products:

The PCR products (amplicons) were separated electrophoretically in 1.5% agarose gel (HiMedia Laboratories Pvt. Ltd., India) containing ethidium bromide (1µl/30 ml agarose) (Sisco Research Ltd., India). Ten µl of 100 bp DNA ladder (MBI Fermentas, Hanover, USA) were loaded in the first slot of the gel. The rest of the wells were loaded with 10µl of PCR products that were mixed with 5µl of 6x loading dye (MBI Fermentas, Hanover, USA). The gel was covered with 1X TBE buffer and electrophoresis was performed in a mini gel electrophoresis (C.B.S and Scientific Co., California) using 100 volts for 40 min using Standard Power Supply (EPS 2A200, Hoefer). DNA bands were visualized and recorded using the gel documentation system (Bio-Rad, USA).

3.3 HISTOPATHOLOGY

The formalin fixed tissues (189) were subjected to overnight washing in running tap water, dehydration in ascending grades of alcohol, clearing in xylene and embedding in paraffin. These paraffin embedded tissues were sectioned at 5µm thickness using semi automatic rotary microtome (Leica, Germany) and stained with routine Haematoxylin and Eosin stain (Culling,1957). Duplicate sections were subjected to special stains:

Methyl green pyronin (Kurnick, 1952; Lyon *et al.*, 1983) and Masson's trichrome stain (Reece, 1996) as per the need.

3.4 IMMUNOHISTOCHEMISTRY

The tissue samples (15) preserved at -20^{0}C subjected to cryo sectioning (Leica, Germany) of 5µm thickness. The sections were lifted on clean grease free slides precoated with Poly-L-Lysine (Sigma, USA) and were subjected for Proliferating Cell Nuclear Antigen (PCNA) staining.

Formalin fixed paraffin embedded sections (75) of 5µm thickness were taken on to Poly-L-Lysine coated slides. Sections were deparaffinised, rehydrated and antigen retrieval was done by boiling in 6M urea solution for 10 min followed by quenching of endogenous peroxidase activity by treating sections with 3% hydrogen peroxide for 10 min and rinsed with 0.1M phosphate buffer saline (PBS) at pH 7.4. After washing in PBS for 2 min. Sections were incubated with 5% normal goat serum (Sigma, G9023) in PBS for 20 min, then rinsed in PBS for 5 min and were incubated with PCNA monoclonal antibodies (1:3000 dilution in PBS with 1% BSA) at 4^{0}C over night in a humidified chamber. After rinsing in the PBS, sections were incubated with biotinylated goat antimouse immunoglobulin IgG (1:20 dilution in PBS) for 30 min and washed twice with PBS. The sections were treated with 1:20 dilution extr avidin peroxidase (Sigma Extr-2) and incubated for 30 min 3-amino-9 ethyl carbazol (AEC) staining kit (Sigma AEC-101) was used for the visualization of immune complexes which gave brick red reaction product. Sections were counter stained with Mayer's Haematoxylin (Sigma MHS-16),

rinsed for 5 min in running tap water and mounted with aqueous glycerol gelatin (Sigma GG-1).

3.4.1 PCNA Index:

The immunoreactivity for PCNA was done by counting the positive cells in 10 randomly selected high power fields (X200) and the mean values were calculated (Berry *et al.*, 2006).

RESULTS

CHAPTER 4

Results

Survey on the incidence of various poultry tumours was carried out. They were confirmed by adopting various diagnostic studies and the results are compiled.

4.1 INCIDENCE

A total of 189 samples from 72 birds at necropsy were collected and were subjected to cytological, molecular, histopathological and immunohistochemical studies (Table 1).

TABLE 1: Details of no. of samples collected and subjected to various diagnostic methods

Source of the collected samples	No. of cases	No. of samples	Cytological studies	Histopathological studies	Molecular studies	Immuno histochemical studies
Dept of Pathology, C.V.Sc, Hyderabad	38	96	38	38	27	25
PDP, Hyderabad	25	54	-	25	24	-
Dept of Microbiology-birds sacrificed	4	17	4	4	4	-
Others (private farms)	5	22	4	5	4	4
Total	72	189	46	72	59	29

The incidence of various neoplastic conditions according to age and breed were shown in tables 2 & 3.

TABLE 2: Incidence of tumours according to age group

S.No	Age group	% of incidence
1	0-8 wks	2 (4%)
2	8-18 wks	23 (46.93%)
3	18-28 wks	13 (26.53%)
4	=28 wks	11 (22.44%)

Total no. of cases – 72; Data available – 49; Data not available – 23

Of 72 number of cases studied, the data of age group was available for 49 and of them the incidence between 0-8 weeks was 2 (4%), between 8-18 weeks was 23 (46.93%), between 18-28 weeks was 13 (26.53%) and above 28 weeks was 11 (22.44%). The incidence was high in birds ranging from 8 to 18 weeks of age and it was least in birds aging less than 8 weeks of age (fig 1).

TABLE 3: Breed wise incidence of tumours

S.No	Breed	% of Incidence
1	White Leghorn	6 (12%)
2	White Leghorn cross breed	3 (6%)
3	Vanaraja	3 (6%)
4	Aseel	5 (10%)
5	Rajasree	23 (46%)
6	Kadaknath	1 (2%)
7	Rajasreee cross breed	9 (18%)

Total no. of cases – 72; Data available – 50; Data not available – 22

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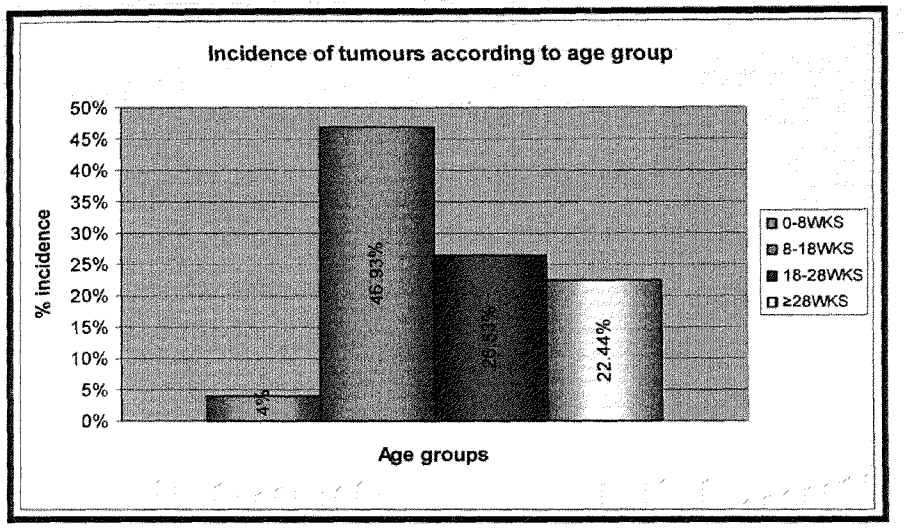

Figure 1: Incidence of tumours according to age group.

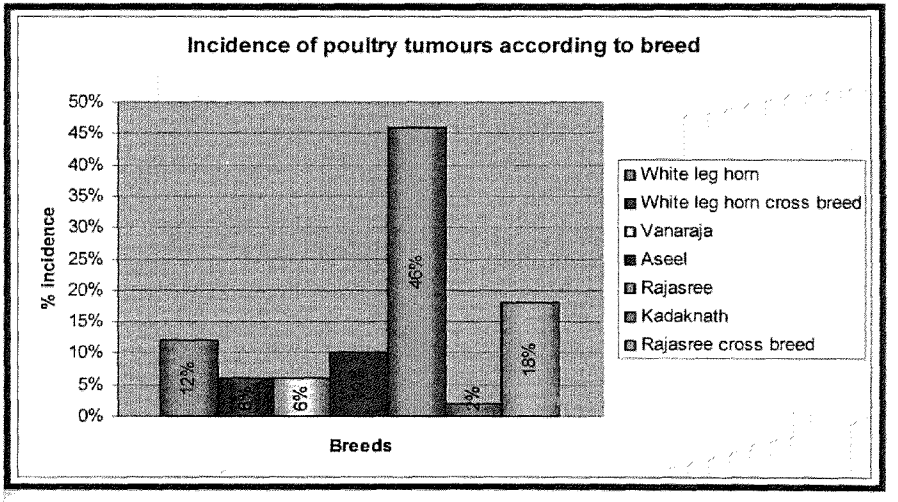

Figure 2: Incidence of tumours according to breed.

Out of 72 cases, the particulars of breed was available for 50 and of them the incidence in White Leghorns was 6 (12%), in White Leghorn crossbreeds was 3 (6%), in Vanaraja was 3 (6%), in Aseel was 5 (10%), in Rajasree was 23 (46%), in Kadaknath was 1 (2%) and it was 9 (18%) in Rajasree cross breeds. The highest incidence was recorded in Rajasree and the lowest in Kadaknath (fig 2).

The incidence of tumours in female birds was 60 (83.33%) and in male birds was 12 (16.66%), resulting more incidence in females.

TABLE 4: Incidence of various types of tumours in poultry

S. No	Various types of poultry tumours	% if incidence
1	Marek's disease	31 (43.05%)
2	Lymphoid leucosis	20 (27.77%)
3	Hepatoma	3 (4.16%)
4	Adenoma	3 (4.16%)
5	Granulosa cell tumour	1 (1.38%)
6	Fibroma	2 (2.77%)
7	Haemangioma	2 (2.77%)
8	Nephroblastoma	1 (1.38%)
9	Osteofibroma	1 (1.38%)
10	Endothelioma	1 (1.38%)
11	Chondroma	1 (1.38%)
12	Other leukotic tumours	6 (8.33%)

The incidence of various types of poultry tumours was recorded as Marek's disease 31 (43.05%), lymphoid leucosis 20 (27.77%), hepatoma 3 (4.16%), adenoma

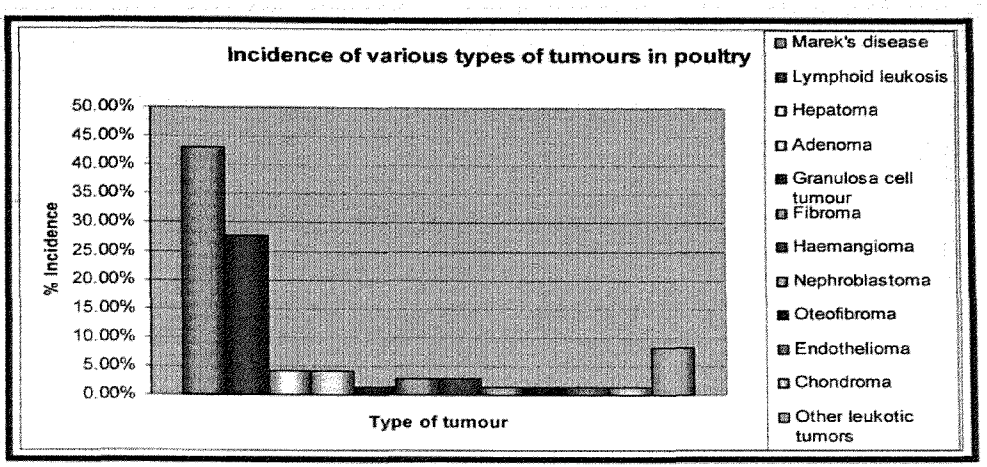

Figure 3: Incidence of various types of tumours in poultry.

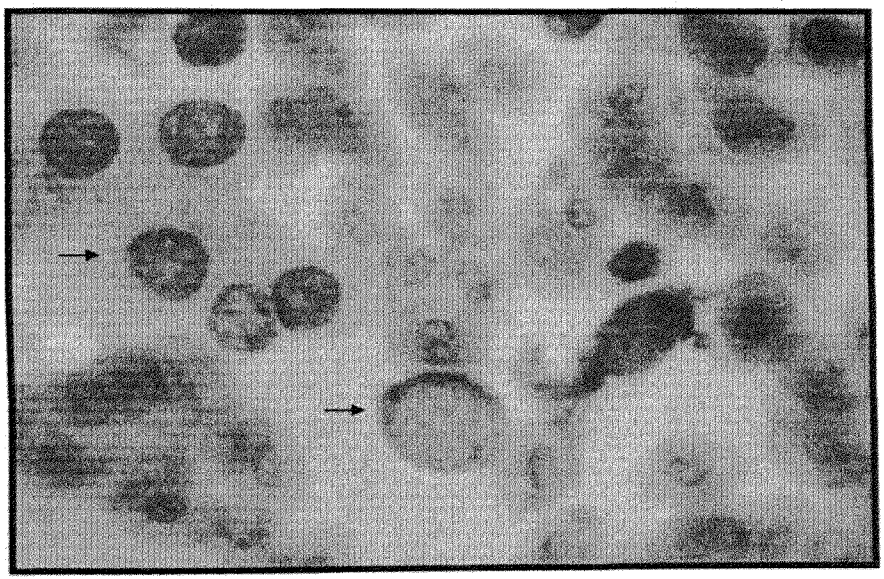

Figure 4: Photograph of impression smear from a liver showing pleomorphic lymphocytes (arrows) with thin rim of cytoplasm and a vesicular nucleus having a fine network of chromatin. Marek's disease (Leishman's stain X1000).

3 (4.16%), granulosa cell tumour 1 (1.38%), fibroma 2 (2.77%), haemangioma 2 (2.77%), nephroblastoma 1 (1.38%), osteofibroma 1 (1.38%), endothelioma 1 (1.38%), chondroma 1 (1.38%) and other leukotic tumours 6 (8.33%). The incidence of Marek's disease was recorded as highest (fig 3).

4.2 CYTOLOGICAL STUDIES

Impression smears of various organs or tissues (90) collected from 46 birds were stained with Leishman's / Giemsa stain (Table 5).

TABLE 5: Incidence of poultry tumours based on the cytological examination.

No	Impression smears of various organs	Total no.	No of samples +ve for MD	No of samples +ve for LL	Others /non specific
1	Liver	28	20	7	1
2	Spleen	12	12	-	-
3	Heart	10	4	6	-
4	Kidney	15	9	6	-
5	Proventriculus	10	8	2	-
6	Ovary	5	4	-	1
7	Lung	3	3	-	-
8	Growths	7	1	-	6
	Total	90	61 (67.78%)	21 (23.33%)	8 (8.88%)

Sixty one (67.78%) smears were diagnosed as Marek' disease depending on the features like lymphoid cells with thin rim of cytoplasm and a vesicular nucleus having a fine network of chromatin and marked pleomorphism (fig 4).

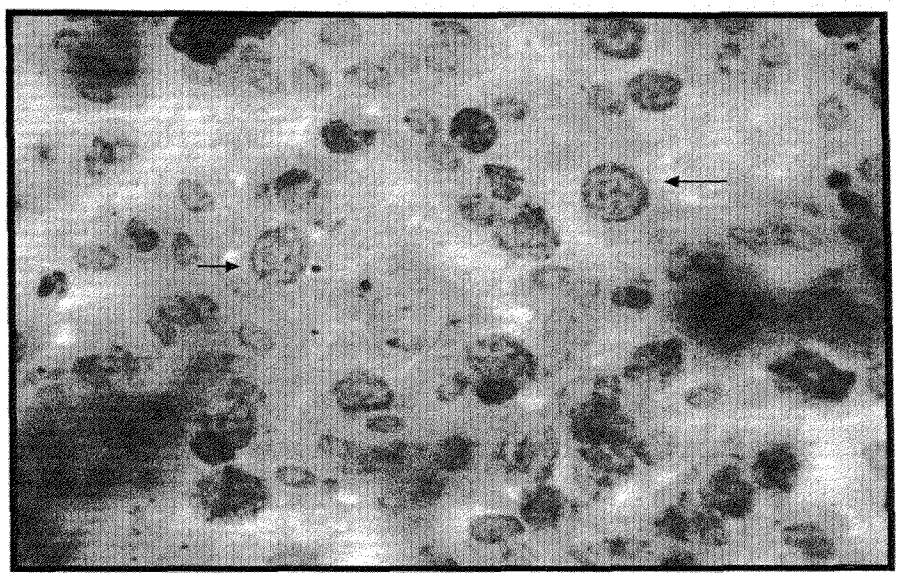

Figure 5: Photograph of impression smear from a liver showing uniform sized lymphocytes (arrows) with thin rim of cytoplasm and a vesicular nucleus having a fine network of chromatin. Lymphoid leucosis (Leishman's stain X1000).

Twenty one (23.33%) impression smears from various organs were diagnosed as lymphoid leucosis as the feature of lymphoid cells were with thin rim of finely granular cytoplasm and stained blue. A clear vesicular nucleus with a very fine and distinctive arrangement of the chromatin and para chromatin. Marked degree of uniformity in size and appearance of neoplastic cells was observed (fig 5).

Eight (8.88%) smears were negative for either MD or LL. The bacterial or fungal infections were ruled out by staining the smears with Gram's and Lactophenol cotton blue stains.

4.2 MOLECULAR STUDIES

Fifty nine tissue samples suspected for MD and LL were subjected for molecular diagnosis. The samples were processed for DNA extraction and PCR was carried out by using two sets of primers that were specific for MDV and ALV. The detail of tissues/ organs collected and studied was given below (Table 6).

TABLE 6: Details of samples subjected for molecular studies

S. No	Tissue/ organ	No of samples	No. confirmed MD	No. confirmed LL	No. negative for MD/ LL
1	Liver	35	19	14	2
2	Spleen	4	1	3	-
3	Heart	3	2	1	-
4	Kidney	9	5	4	-
5	Ovary	6	3	2	1
6	Growths	1	-	-	1
7	Trachea	1	-	1	-
	Total	59	30 (50.85%)	25 (42.37%)	4 (6.78%)

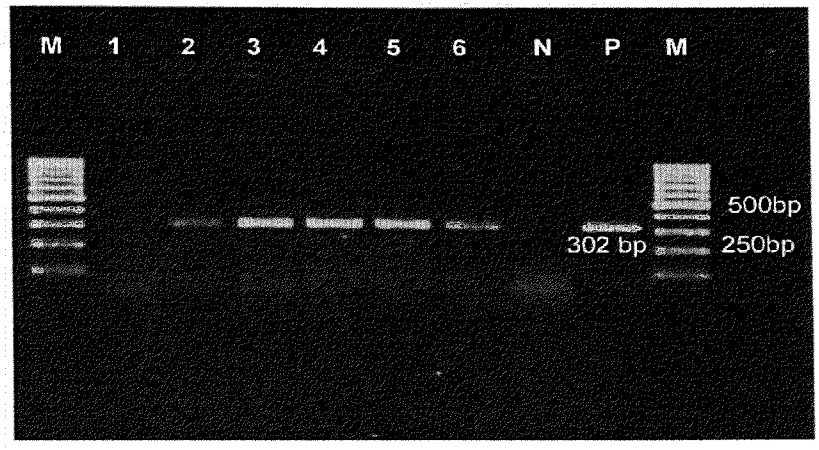

Figure 6: 132 bp tandem repeat sequence specific PCR using M1 and M2 primers for detection of MDV.

Lane M	- 100 bp DNA ladder
Lane 1	- Sample from private farms, negative for MDV
Lanes 2 to 6	- Samples from PDP, positive for MDV
Lane N	- Negative control
Lane P	- Positive control

PCR products were analyzed on 1.5% agarose gel containing 5µg/ ml ethidium bromide.

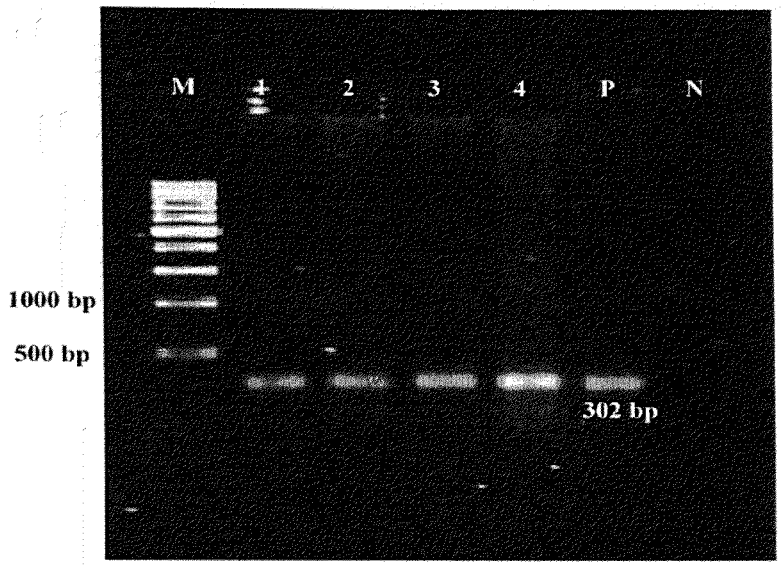

Figure 7: 132 bp tandem repeat sequence specific PCR using M1 and M2 primers for detection of MDV.

Lane M	- 1 kb DNA ladder
Lane 1 to 3	- Samples from experimental birds, positive for MDV
Lanes 4	- Sample from experimental birds, positive for MDV
Lane P	- Positive control
Lane N	- Negative control

PCR products were analyzed on 1.5% agarose gel containing 5µg/ ml ethidium bromide

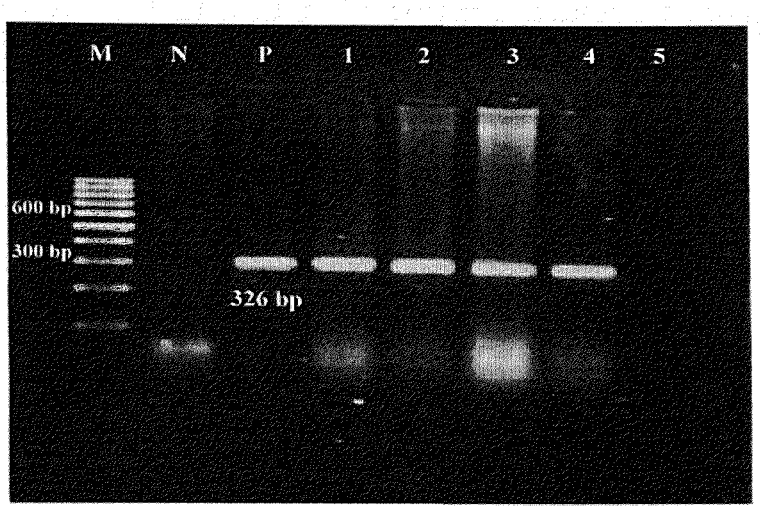

Figure 8: *Pol* gene specific PCR using AD1 and H5 primers for detection of ALV.

Lane M - 100 bp DNA ladder

Lane N - Negative control

Lane P - Positive control

Lanes 1 to 4 - Samples from necropsy at Dept. of Pathology, positive for ALV

Lane 5 - Sample from negative for ALV

PCR products were analyzed on 1.5% agarose gel containing 5µg/ ml ethidium bromide

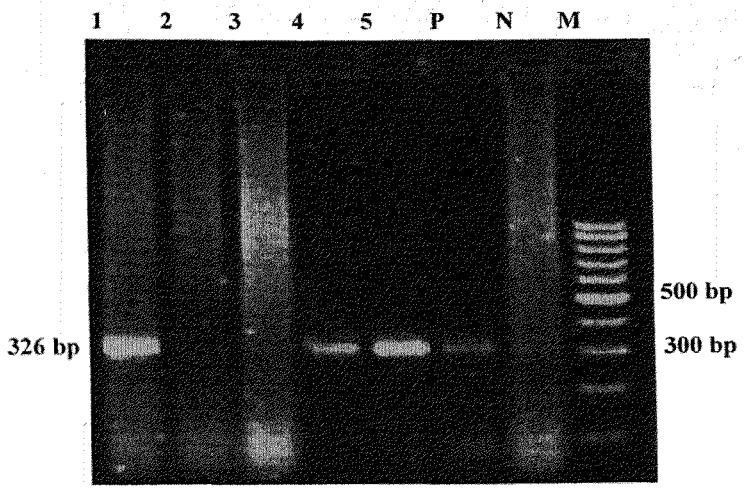

Figure 9: *Pol* gene specific PCR using AD1 and H5 primers for detection of ALV.

Lane 1	- Samples from necropsy, Dept of Pathology, positive for ALV
Lanes 2, 3	- Sample from PDP, negative for ALV
Lanes 4, 5	- Samples from private farms, positive for ALV
Lane P	- Positive control
Lane N	- Negative control
Lane M	- 100 bp DNA ladder

PCR products were analyzed on 1.5% agarose gel containing 5µg/ ml ethidium bromide

The DNA from 30 (50.85%) out of 59 samples were amplified by MD specific M1 and M2 primers and produced an amplicon approximately of 302 bp size (fig 6, 7) characteristic of *132 bp* repeats of genome of MDV.

The DNA from 25 (42.37%) samples was amplified with ALV specific AD1 and H5 primers produced an amplicon approximately of 326 bp size (fig 8, 9) characteristic for *Pol* gene of ALV which was conserved among ALV subgroups A, B, C, D & E.

The DNA from remaining 4 (6.78%) samples was neither amplified for MDV nor for ALV with any of the above primers

4.3 GROSS LESIONS

A total of 72 suspected poultry tumour cases were collected and the gross lesions were described.

TABLE 7: Incidence of poultry tumours based on gross lesions

S. No	Cases suspected for	% of incidence
1	Marek's disease	18 (25%)
2	Lymphoid leucosis	45 (62.5%)
3	Haemangioma	2 (2.77%)
4	Fibroma	7 (9.72%)
	Total	72

TABLE 8: Incidence of poultry tumours based on gross lesions of various organs

S. No	Tissue/ organ	No. of samples	MD	LL	Others
1	Liver	53	14	38	Haemangioma (1)
2	Spleen	35	18	17	-
3	Heart	31	19	12	-
4	Kidney	34	5	29	-
5	Proventriculus	17	9	8	-
6	Ovary	8	4	3	Haemangioma (1)
7	Lung	3	-	3	-
8	Growths	7	-	-	Fibroma (7)
9	Trachea	1	-	1	-
	TOTAL	189	69 (36.50%)	111 (58.73%)	9 (4.76%)

Marek's Disease:

Out of the 72 cases 18 cases were suspected for Marek's disease based on the gross lesions of livers (14), spleen (1), hearts (2), kidneys (5) and ovaries (3).

Grossly, on the liver enlarged friable with diffusely distributing greyish white nodules (fig 10) of variable sizes few nodules coalesced with each other forming big nodules. Few nodules were poorly demarcated. Cut surface revealed that the parenchyma was involved. Spleen was grossly enlarged to 3-4 times than its normal size with a diffuse white or greyish discolouration (fig 11). Hearts were pale, enlarged

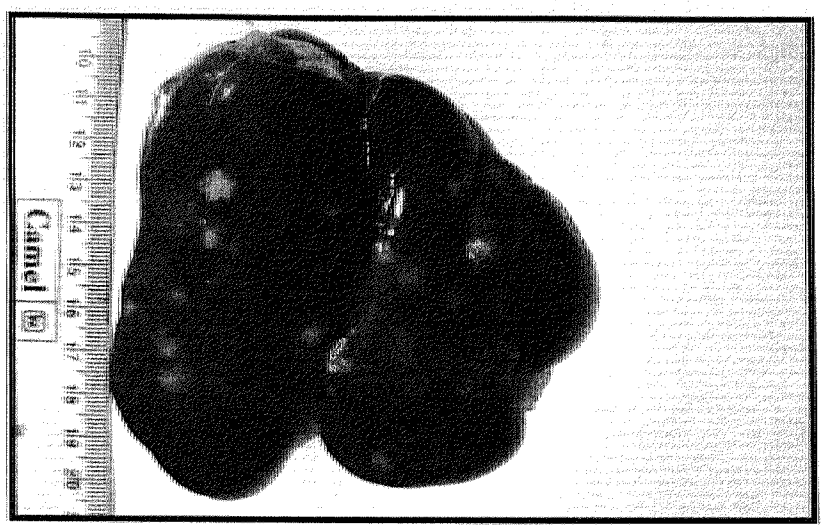

Figure 10: Photograph of liver with diffuse multiple white nodular growths. Marek's disease.

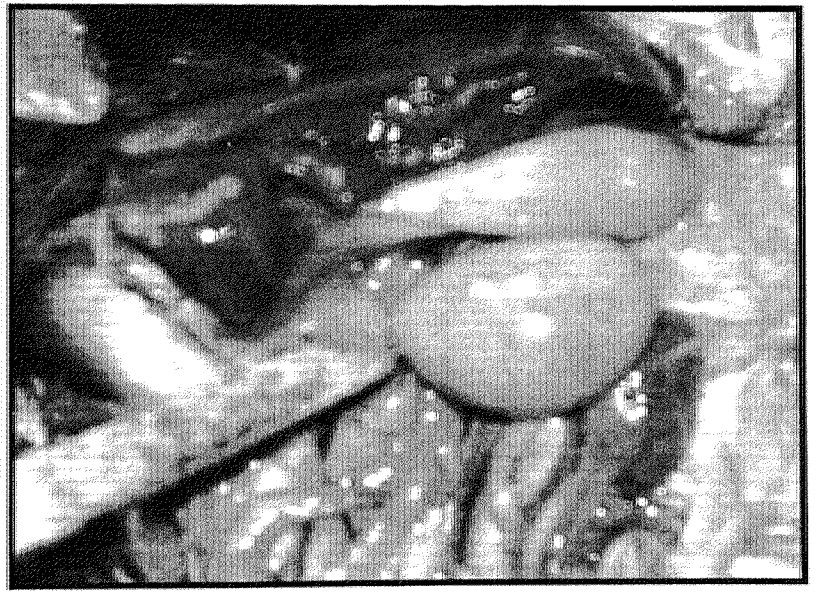

Figure11: Photograph showing gross enlargement of spleen and proventriculus. Marek's Disease.

and had single or multiple nodular tumours in the myocardium and pinpoint miliary whitish foci in the epicardium. The tumours were soft, smooth, greyish and creamy white in colour. Kidneys were enlarged, pale and had pinpoint whitish nodules. Proventriculi were grossly enlarged, walls got thickened and firm (fig11). Affected ovaries were greyish white in colour, soft and fleshy in consistency and had moderate to marked enlargement (fig 12).

Lymphoid leucosis:

Forty five cases were suspected for lymphoid leucosis depending on the gross lesions of livers (38), spleens (17), hearts (12), kidneys (29), proventriculi (8), ovaries (3), lungs (3) and trachea (1) were observed.

The growths observed in livers occurred in three patterns viz. *nodular, miliary* and diffuse. Livers with diffuse involvement were uniformly enlarged, slight greyish in colour and were friable (fig 13). Livers with nodular form showed lymphoid tumours, single or multiple, variable sizes and were spherical (fig 14). Livers with miliary form showed numerous small nodules with uniform distribution of entire parenchyma. The tumours were soft, smooth and glistening. Cut surface appeared greyish to creamy white and had areas of necrosis. Sometimes diffuse form along with nodular or miliary forms was noticed.

Spleens were grossly enlarged with miliary greyish white nodules on them (fig 15) or with diffuse leucosis (fig 16). Few spleens also exhibited petechiae along with tumour nodules. Hearts showed distinct multiple nodular tumour masses without any gross enlargement. The tumour masses were soft and white in colour involving both epicardium and myocardi

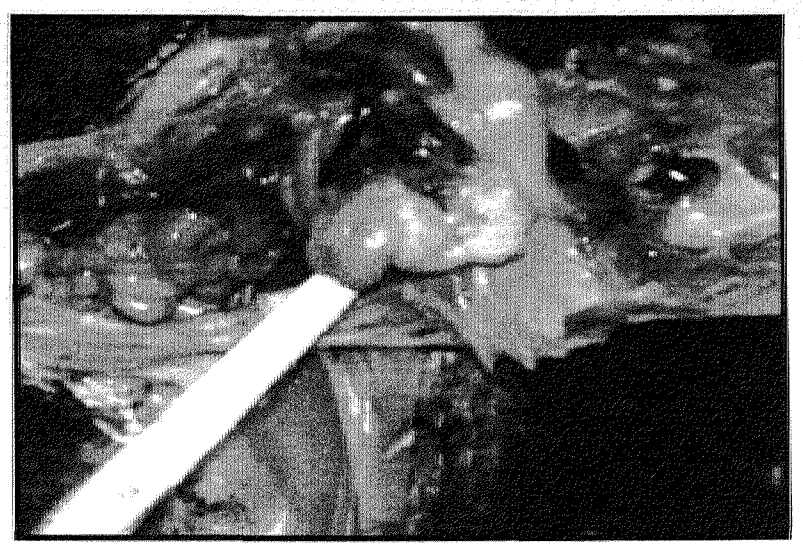

Figure 12: Photograph showing grayish white enlarged ovaries having cauliflower like appearance. Marek's disease.

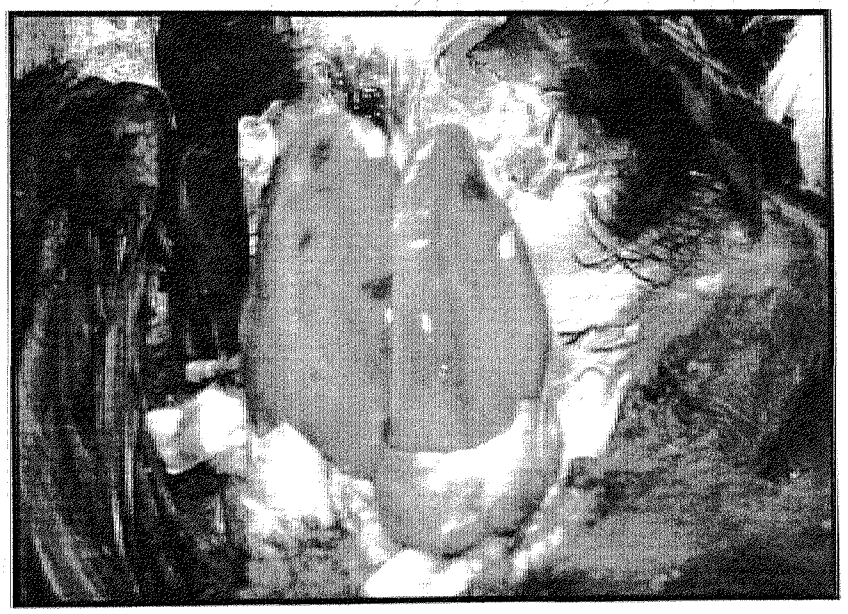

Figure 13: Photograph showing gross enlargement of liver with diffuse leucosis. Diffuse form of Lymphoid Leucosis

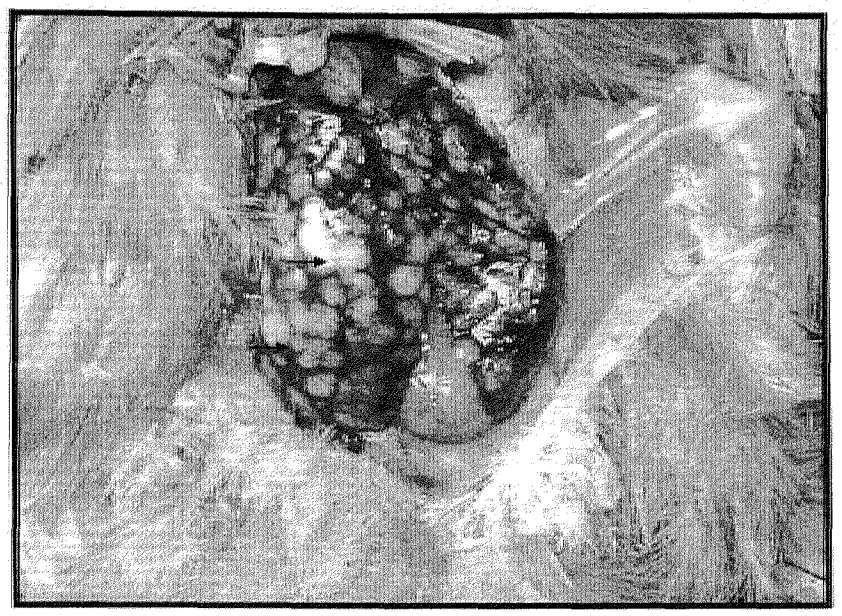

Figure 14: Photograph showing liver with multiple grayish white nodules of variable sizes. Note the smaller nodules coalescing with each other to form bigger nodules (arrows). Nodular form of Lymphoid Leucosis.

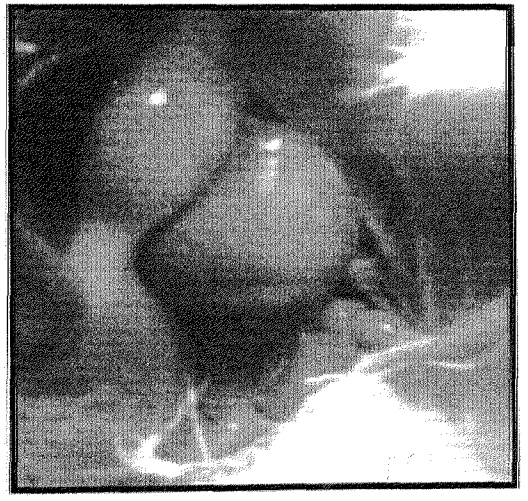

Figure 15: Photograph showing grossly enlarged spleen with grayish white nodules. Lymphoid leucosis.

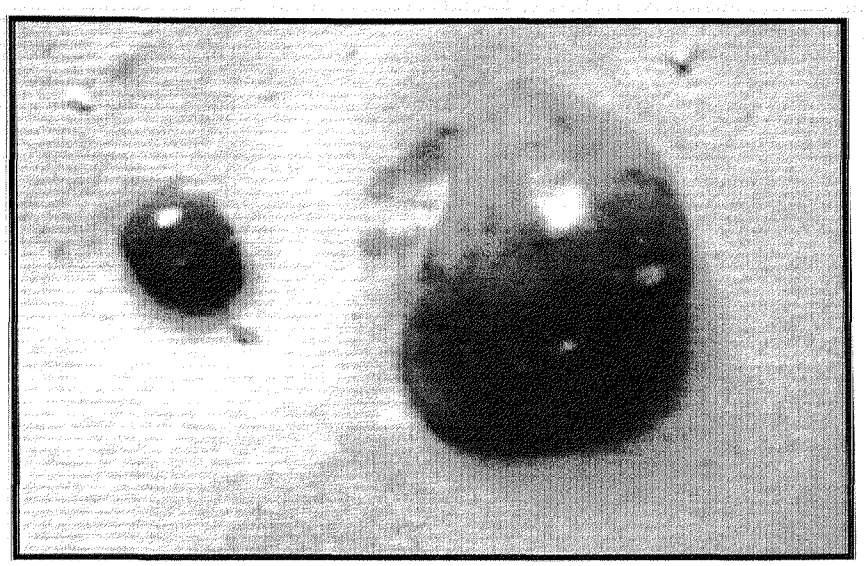

Figure 16: Photograph showing gross enlargement of spleen many times to its normal size with diffuse Lymphoid Leucosis.

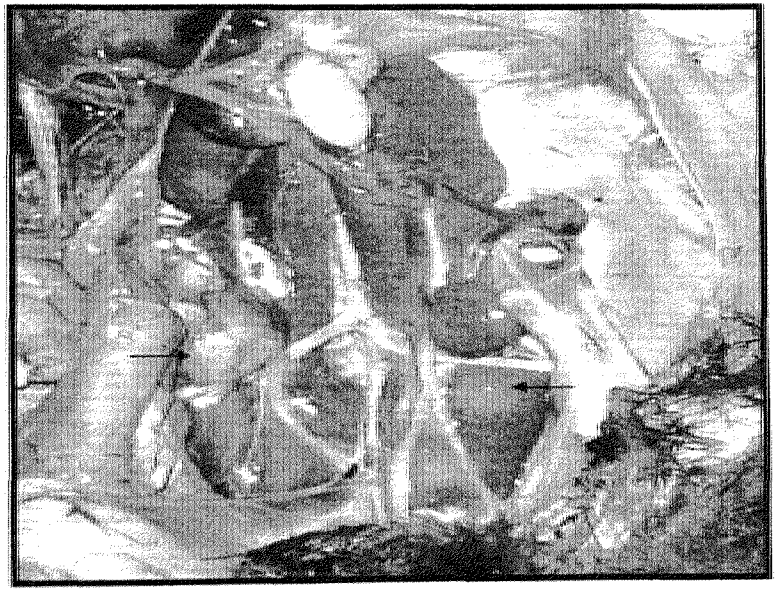

Figure 17: Photograph of kidney showing poorly demarcated lymphoid nodules (arrows). Lymphoid Leucosis.

Kidneys were greatly enlarged, pale with poorly demarcated big whitish nodules (fig 17). Few kidneys were diffusely leukotic. Proventriculi were grossly enlarged with single or multiple white nodules on them. Ovaries were grossly enlarged, greyish white in colour and had lobulated tumour masses or cauliflower like appearance. Lungs affected were congested, had multiple lymphoid nodular tumour masses. Trachea was slightly thickened with areas of leucosis.

Haemangioma:

The liver was enlarged with reddish specks on both external surface and also on cut sections. The ovary was completely red and was enclosed in a capsule. Cut surface had lamellated appearance and on incision blood oozed out on cutting.

Growths:

A total of 7 suspected growths were collected and were suspected for fibroma. Three growths were located at the junction of proventriculus and duodenum and they were round, firm, hard in consistency, and of considerable size. The cut surface was greyish and revealed bundles of fibrous tissue running across section.

Two growths were located one in the gizzard and another on the small intestine in a same bird. Both were alike as round firm, hard masses with central area of ulceration and necrosis. They were about 1cm in diameter. Cut surface was greyish white in colour with presence of fibrous tissue.

One growth was located at the shank region of right leg of a female bird. The leg was diffusely enlarged, ulcerated with areas of necrosis (fig 18). The growth was hard, pale in colour and was difficult to cut. One more growth was located on the skin

of head region. It was a small, round nodular mass measuring about 0.5 cm in diameter, immovable, firm and hard in consistency.

4.4 HISTOPATHOLOGICAL STUDIES

A total of 189 tissue samples from 72 cases were subjected to histopathological studies (Tables 9 &10).

TABLE 9: Incidence of poultry tumours based on histopathology

S. No	Condition/ Type of tumour	% if incidence
1	Marek's disease	31 (43.05%)
2	Lymphoid leucosis	20 (27.77%)
3	Hepatoma	3 (4.16%)
4	Adenoma	3 (4.16%)
5	Granulosa cell tumour	1 (1.38%)
6	Fibroma	2 (2.77%)
7	Haemangioma	2 (2.77%)
8	Nephroblastoma	1 (1.38%)
9	Osteofibroma	1 (1.38%)
10	Endothelioma	1 (1.38%)
11	Chondroma	1 (1.38%)
12	Other leukotic tumours	6 (8.33%)
	TOTAL	72

TABLE 10: Tissue / Organ wise incidence of poultry tumours based on histopathology

S. No	Organ/ Tissue	No. of samples	Confirmed MD	Confirmed LL	Others
1	Liver	53	39	10	Haemangioma-1 Hepatoma- 3
2	Spleen	35	22	12	Endothelioma- 1
3	Heart	31	21	10	-
4	Kidney	34	23	10	Nephroblastoma- 1
5	Proventriculi	17	9	5	Adenoma- 3
6	Ovary	8	3	3	Haemangioma- 1 Granulosa cell tumour- 1
7	Lung	3	2	1	-
8	Growths	7	1	2	Fibroma- 2 Chondroma- 1 Osteofibroma- 1
9	Trachea	1	-	1	-
	TOTAL	189	120 (63.49%)	55 (29.10%)	14 (7.4%)

Marek's Disease:

Of 72 cases, histologically 31 (43.05%) were confirmed as Marek's disease. Tissue samples included livers (39), spleens (22), hearts (21), kidneys (23),

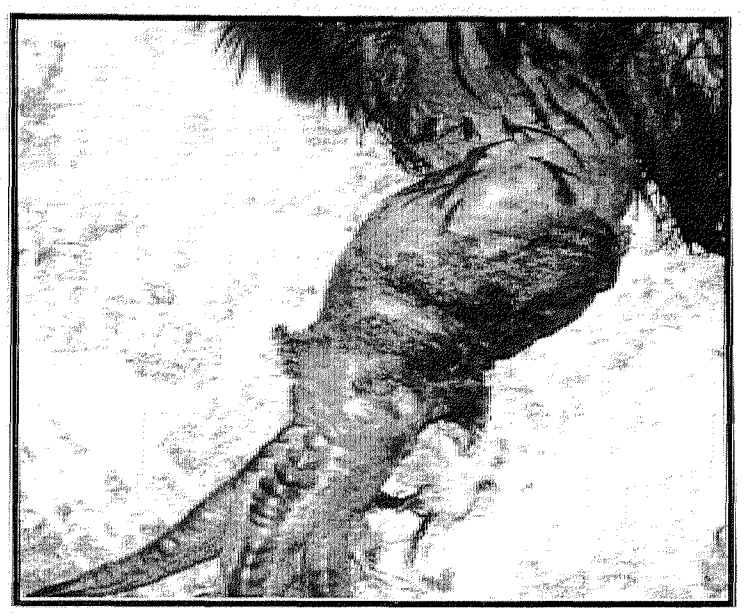

Figure 18: Photograph showing growth at leg region.

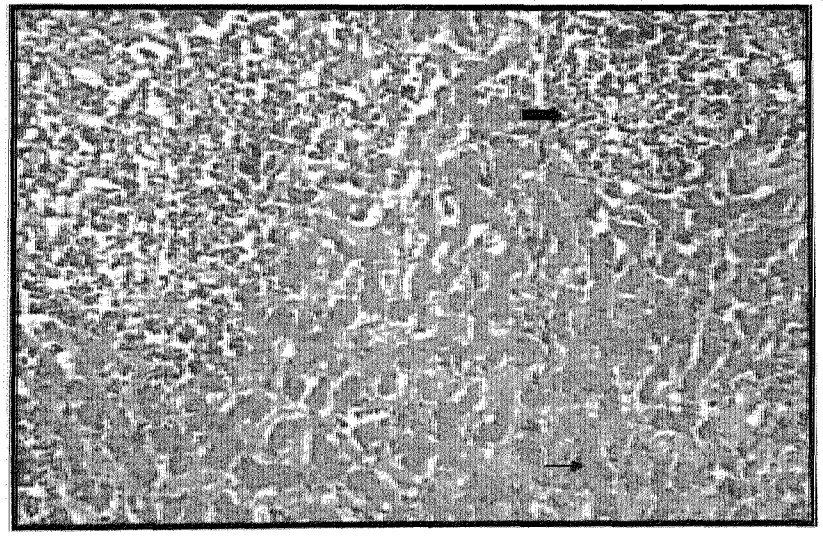

Figure 19: Microphotograph of the liver section showing lymphoid nodule formed by pleomorphic lymphoid cells (block arrow). Hepatocytes showing marked fatty change and moderate degenerative changes (line arrow). Marek's disease (H&E, X200).

proventriculi (9), ovaries (3), lungs (2) and one growth at junction of proventriculus and duodenum.

The liver sections revealed both diffuse and focal infiltration of pleomorphic lymphoid cells including small, medium and large lymphocytes, lymphoblasts and reticulum cells (fig 19). In diffuse type, the entire hepatic tissue was infiltrated by solid sheets of lymphoid cells compressing and obliterating the normal structure of the organ. The focal type was characterized by infiltration of small to large foci of lymphoid cells in the hepatic tissue. In both diffuse and focal type, the blood vessels were thickened and enlarged by the presence of tumour emboli (fig20). In few focal forms, the infiltration of neoplastic lymphoid cells were especially perivascular, indicated an early stage of metastasis. In few sections, in addition to neoplastic changes, infiltration of heterophils was noted but in many cases, variable degrees of degenerative changes were observed in hepatocytes. The sections were subjected to methyl green pyronin stain and found to be negative (fig 21).

Histologically, spleen revealed depletion and degeneration of lymphocytes in and around germinal centers and washed out appearance (fig 22). The splenic vessels (fig 23) and trabeculae (fig 24) in few cases were thickened and were subjected to Masson's trichrome stain (fig 25, 26). Large transformed or proliferating lymphoblasts were noticed around vessels was the characteristic feature and confirmed as MD (fig 27).

Proliferation and infiltration of pleomorphic lymphoid cells resulted separation or even rupture of cardiac muscle fibre bundles in the myocardium (fig 28, 29). Infiltration of lymphoid cells was both diffuse and focal nodular observed in epicardium and endocardium in few sections.

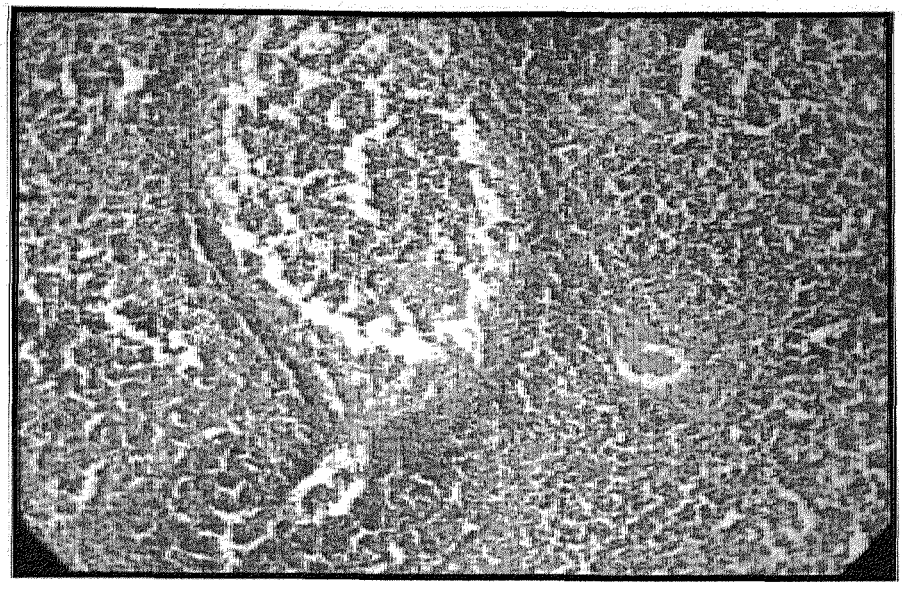

Figure 20: Microphotograph of the liver section showing vessel with tumour embolus consisting of pleomorphic lymphoid cells indicating metastasis. Marek"s disease (H&E, X200).

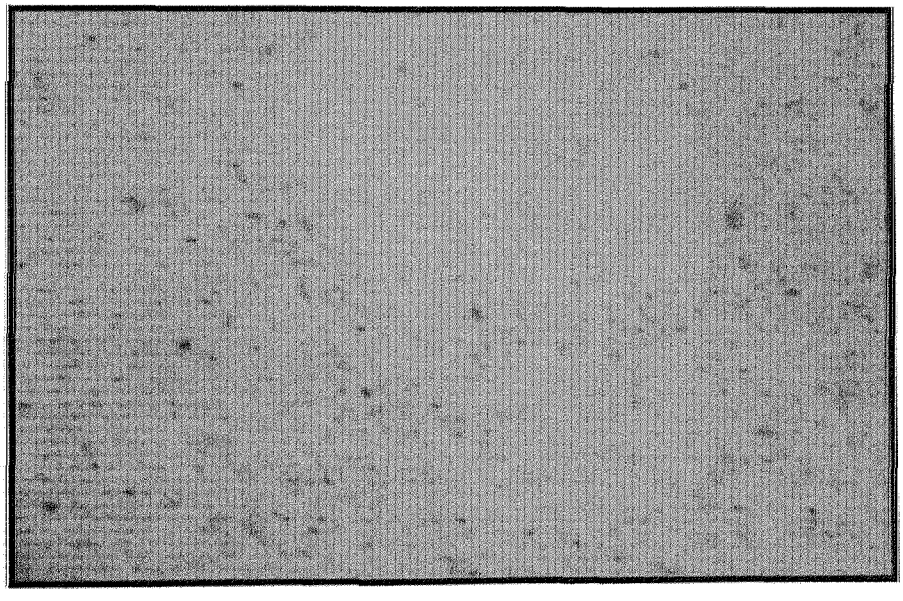

Figure 21: Microphotograph of spleen section showing negative staining with methyl green pyronin. Marek"s disease (X400).

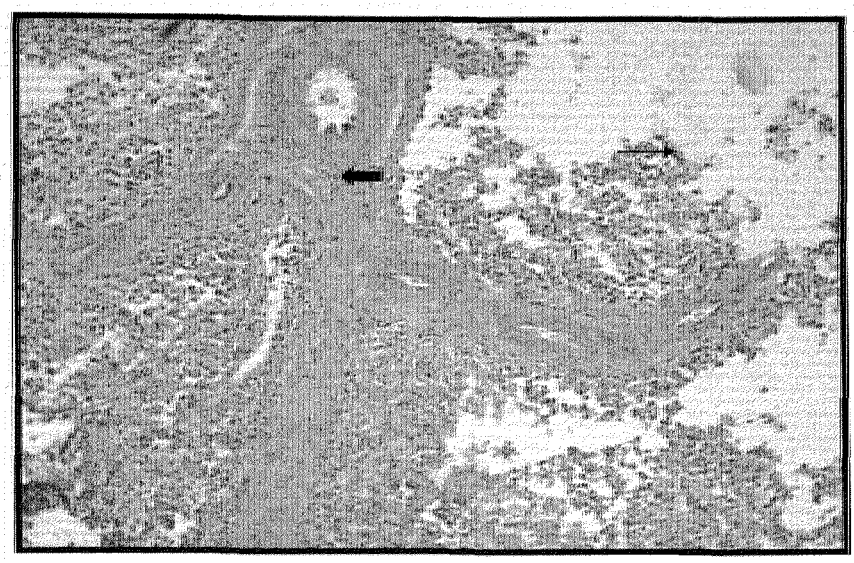

Figure 22: Microphotograph of the section of spleen showing thickened and proliferating blood vessels with transformed cells around them indicate malignancy (block arrow). Washed out appearance of the organ (line arrow). Marek's disease (H&E, X200).

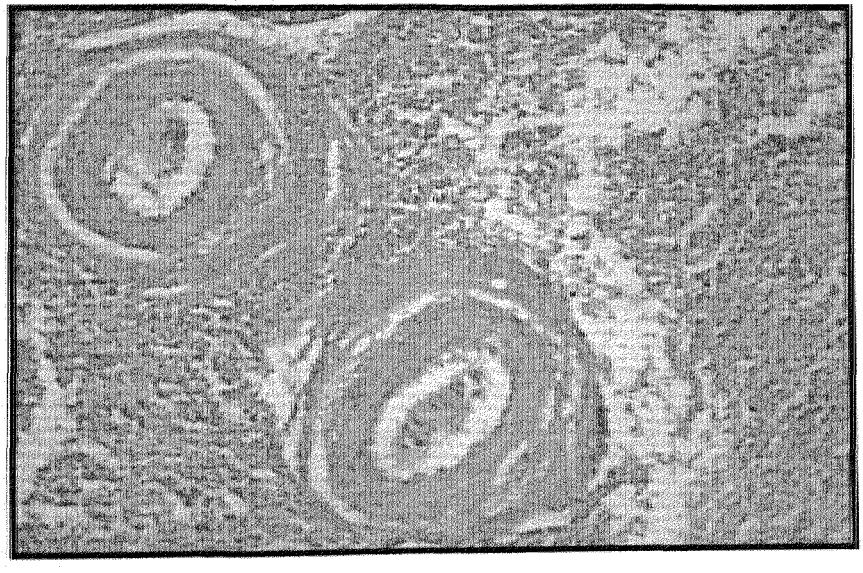

Figure 23: Microphotograph of the section of spleen showing thickened spleenic vessels and depletion of lymphocytes. Marek's disease (H&E, X200)

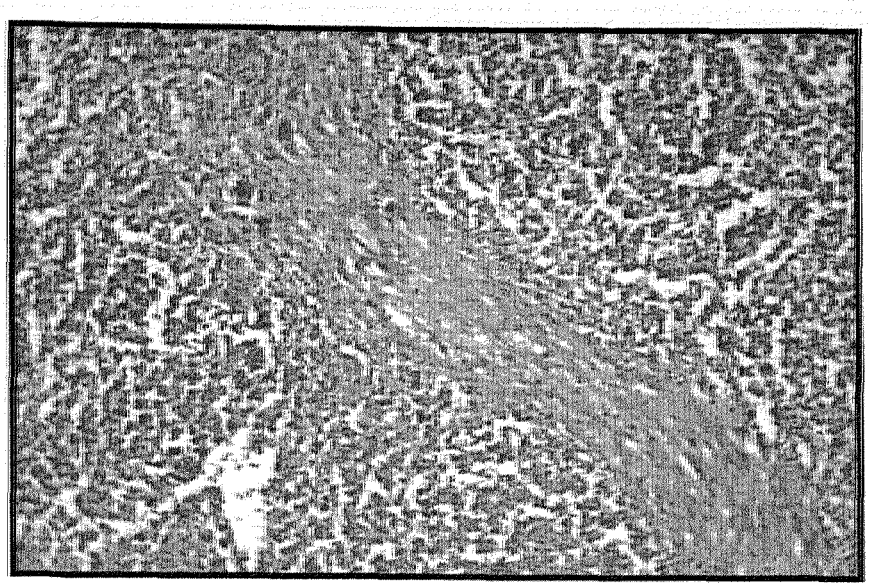

Figure 24 : Microphotograph of the section of spleen showing thickened trabaculae. Marek"s disease (H&E, X200).

Figure 25: Microphotograph of the section of spleen showing thickened blood vessels. Marek"s disease (Masson"s trichrome stain, X1000).

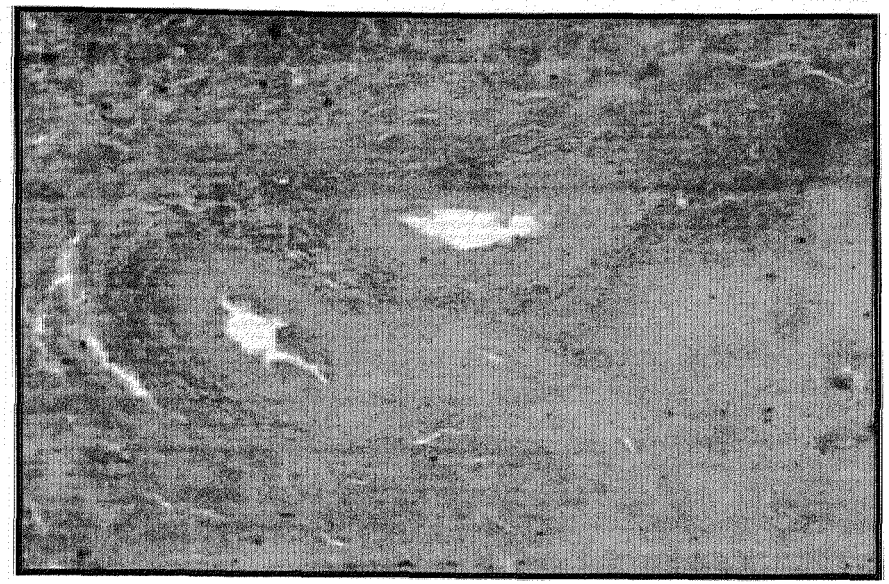

Figure 26: Microphotograph of the section of spleen showing thickened blood vessels. Marek's disease (Masson's trichrome stain, X200).

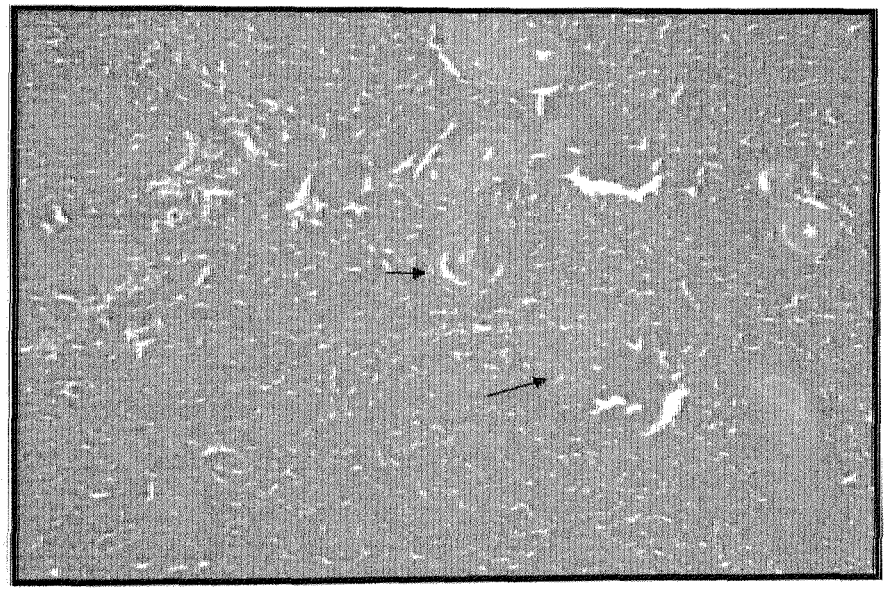

Figure 27: Microphotograph of spleen showing numerous blood vessels and proliferation of transformed lymphoid cells (*arrows*). Marek's disease (H&E, X200).

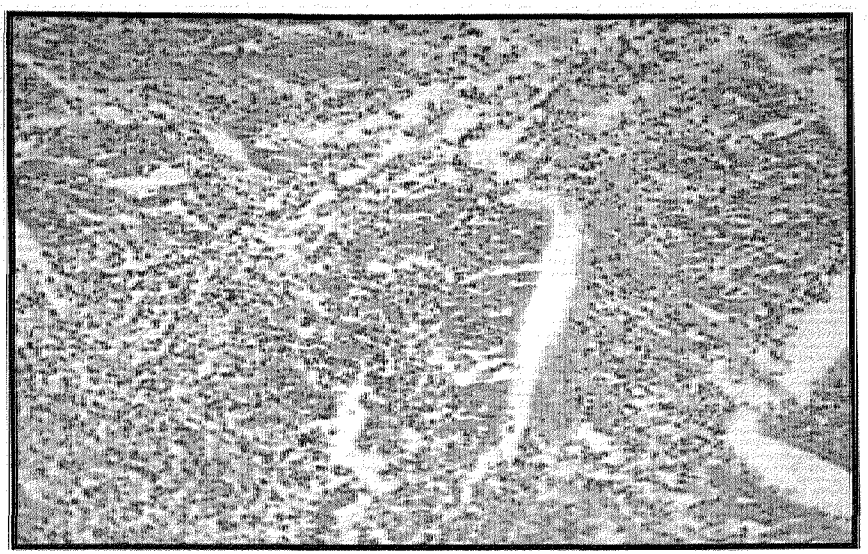

Figure 28: Microphotograph of the section of heart showing diffuse infiltration of pleomorphic lymphoid cells causing separation and rupture of myofibrils. Marek's disease (H&E, X200).

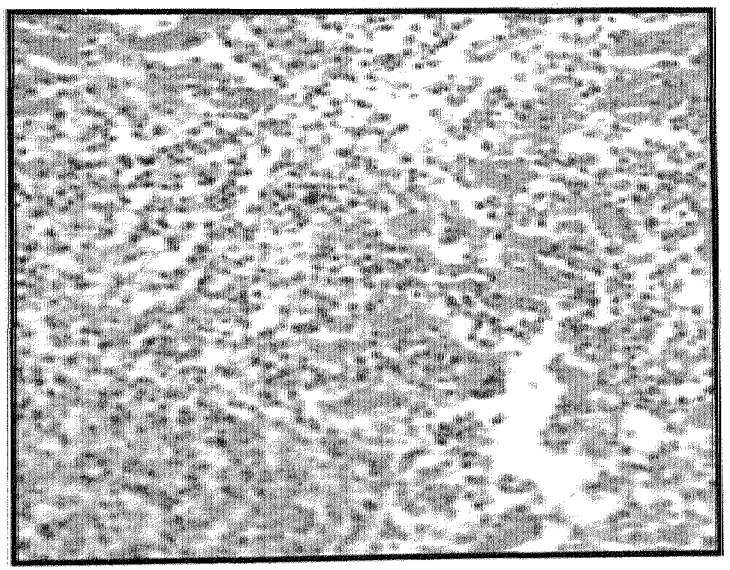

Figure 29: High resolution of figure 28 showing clear pleomorphic cells. Marek's disease (H&E, X400).

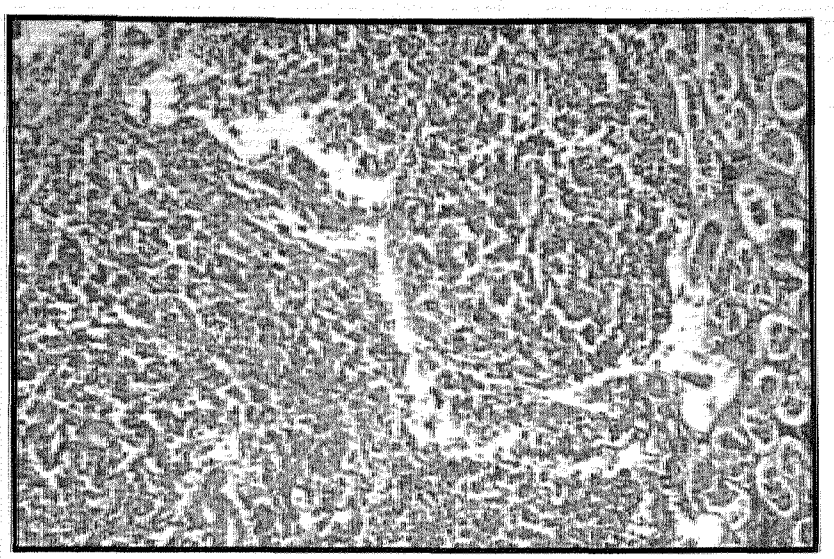

Figure 30: Microphotograph of the section of kidney showing degeneration and desquamation of tubular epithelium, infiltration of lymphoid cells compressing and replacing the renal tissue. Marek's disease (H&E, X200).

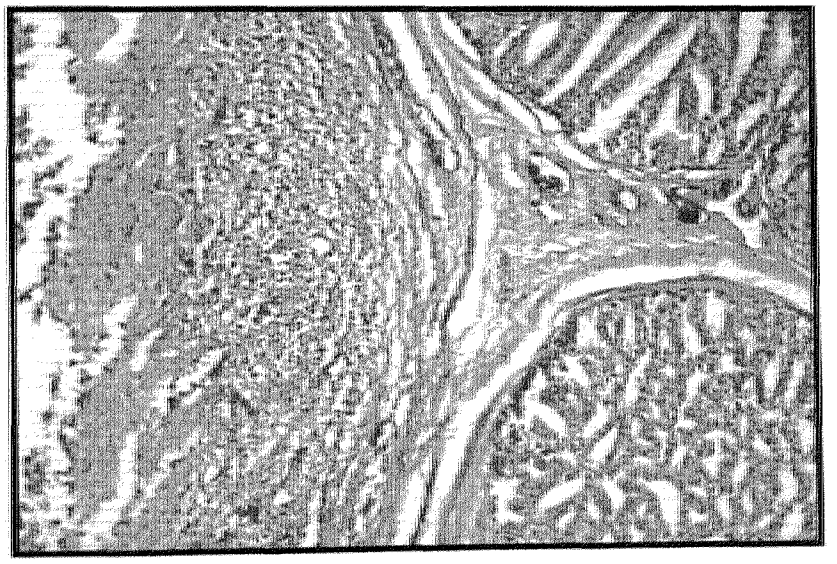

Figure 31: Microphotograph of the section of proventriculus showing infiltration of pleomorphic lymphoid cells in the mucosa and fibrous tissue proliferation. Marek's disease (H&E, X200).

The sections of kidney revealed similar features as observed in the above described organs. Heavy infiltration of lymphoid cells sometimes made difficult to identify the normal architecture of the organ (fig 30). Enlarged and distended vessels with tumour emboli were noted.

The sections of proventriculus showed hyperplasia of lymphoid nodules and infiltration of lymphoid cells in submucosa and within and in between the glands along with considerable amount of fibrous tissue (fig 31, 32). Glandular hyperplasia was noted in few sections. Areas of necrosis were observed in the central portion of the glands.

The sections of ovary exhibited extreme infiltration of lymphocytes displacing the entire ovarian tissue and only a few scattered follicles were discerned.

Lymphoid leucosis:

Histologically, 20 cases (27.77%) were diagnosed as lymphoid leucosis. A total of 55 (29.10%) tissue samples of various organs viz. livers (10), spleens (12), hearts (10), kidneys (10), proventriculi (5), ovaries (3), lungs (1), trachea (1) and growths (2) were confirmed as LL.

Extensive proliferation and infiltration of lymphoid cells was recorded in liver sections. The histological pattern of livers that were nodular type revealed, nodules that were usually surrounded by a band of fibroblast like cells and consisted of aggregates of uniform sized large lymphoid cells or lymphoblasts of early developmental stage (fig 33). The cytoplasmic membrane was poorly defined with much basophilic cytoplasm and a vesicular nucleus where margination and clumping of the chromatin with presence of one or more conspicuous acidophilic nucleoli was observed. The proliferating tumour cells compressed the sinusoids and displaced

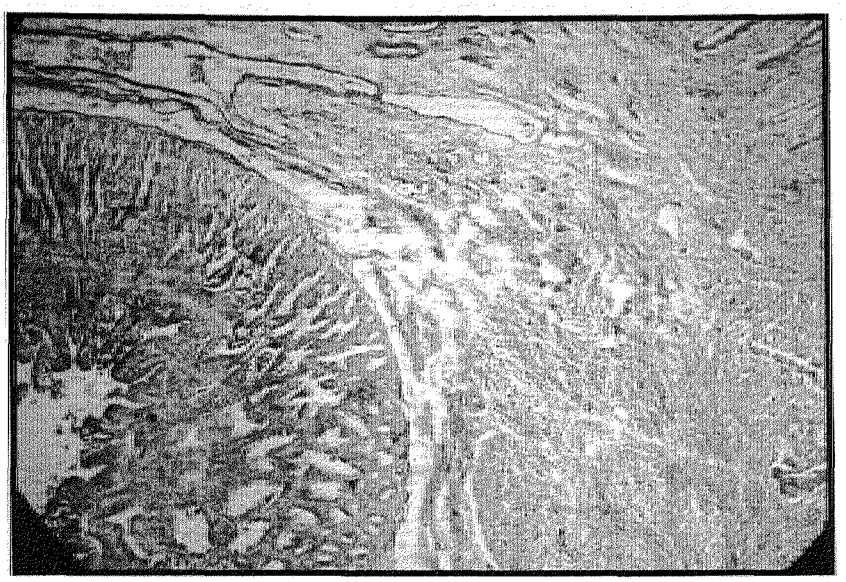

Figure 32: Microphotograph of the section of proventriculus showing fibrous tissue proliferation (Masson's trichrome stain, X100).

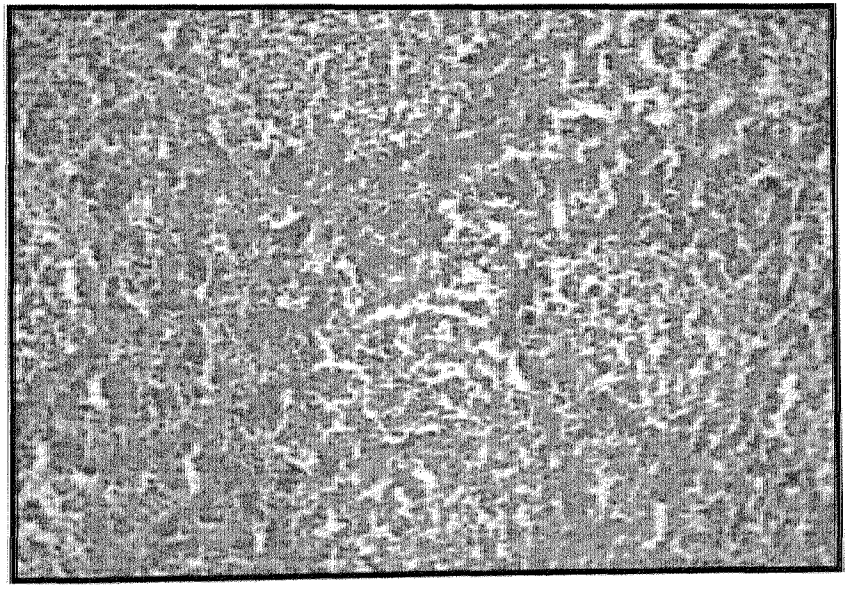

Figure 33: Microphotograph of the section of liver showing diffuse infiltration and lymphoid nodule formation by uniform sized lymphoid cells. Lymphoid leucosis (H&E, X200).

them. In miliary and diffuse types, the histological pattern of the tumour cells was found to be small foci or follicles, coalescing with each other and formed bigger nodules. The hepatic vessels revealed tumour embolus, which was composed of neoplastic cells along with erythrocytes and proliferation of uniform lymphoid cells around the blood vessel indicating early metastasis in few sections (fig 34, 35). Variable degrees of degenerative changes were observed in hepatocytes.

Microscopically, spleen revealed clear lymphoid nodule formation, periarteriolar nodules composed of large lymphoblasts with clear vesicular nucleus. In few sections, the lymphoid transformation in the periphery, underneath the capsule and depletion of lymphocytes in and around germinal centres and thickened blood vessels were observed. The sections stained methyl green pyronin stain revealed red stained RNA in large amounts in the cytoplasm of most of the lymphoid cells (fig 36).

Diffuse accumulation and proliferation of lymphoid cells among myocardial fibres and in few sections the follicles formation was by neoplastic lymphoid cells was observed (fig 37). The morphology of tumour cells was similar to that observed in liver sections.

Infiltration and proliferation of lymphoid cells in kidney sections were noted (fig 38). Marked degree of degenerative changes in tubules in almost all the sections ranged from cloudy swelling, fatty change to *desquamation and necrosis* of tubular epithelium. In few sections the vessels were dilated with presence of tumour emboli.

Pleomorphism in the lymphoid cell population in proventriculi was not observed but there was diffuse lymphoid cell infiltration in the submucosa, in between glands and sometimes in serosa too and hyperplastic changes in glandular epithelium was observed (fig 39).

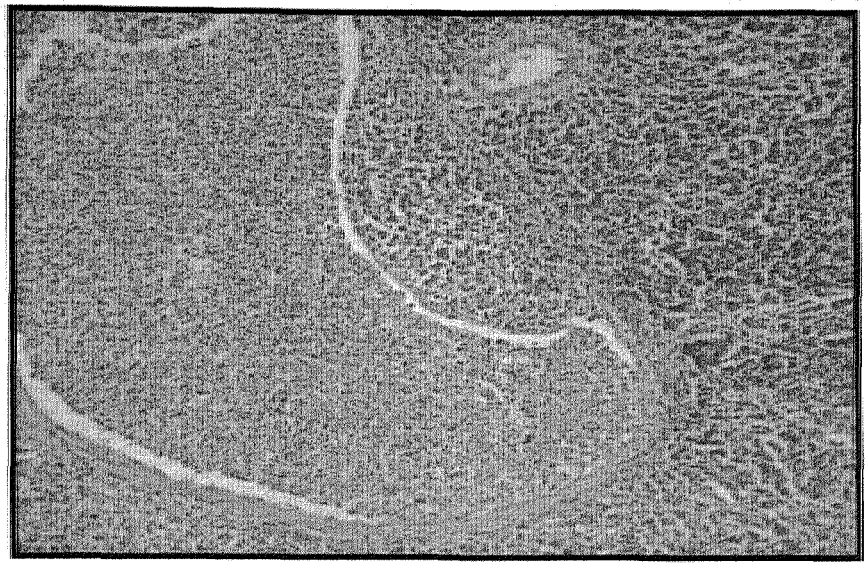

Figure 34: Microphotograph of the liver section showing tumour embolus adhering to the blood vessel consisting of large lymphoblasts. Lymphoid Leucosis (H&E, X200).

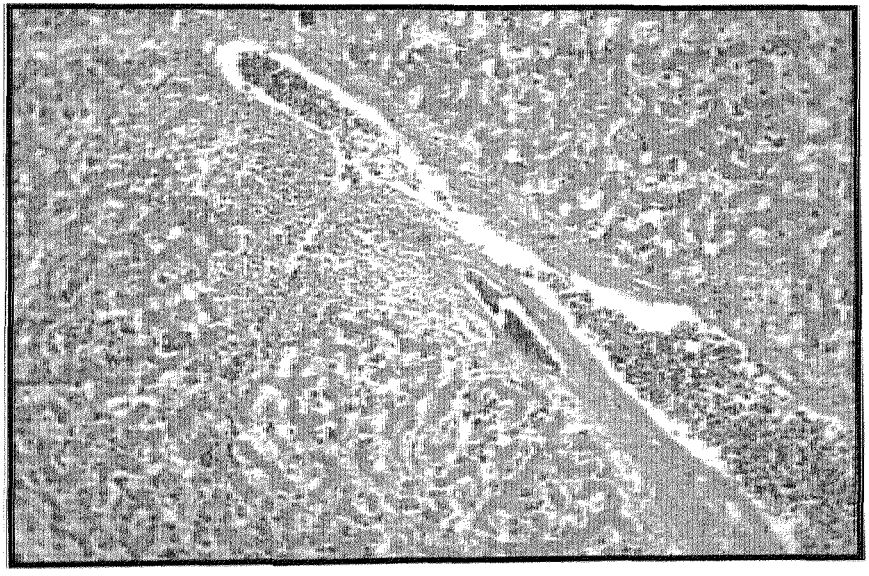

Figure 35: Microphotograph of the section of liver showing proliferation of uniform lymphoid cells around the blood vessel indicating early metastasis. Lymphoid Leucosis (H&E, X200).

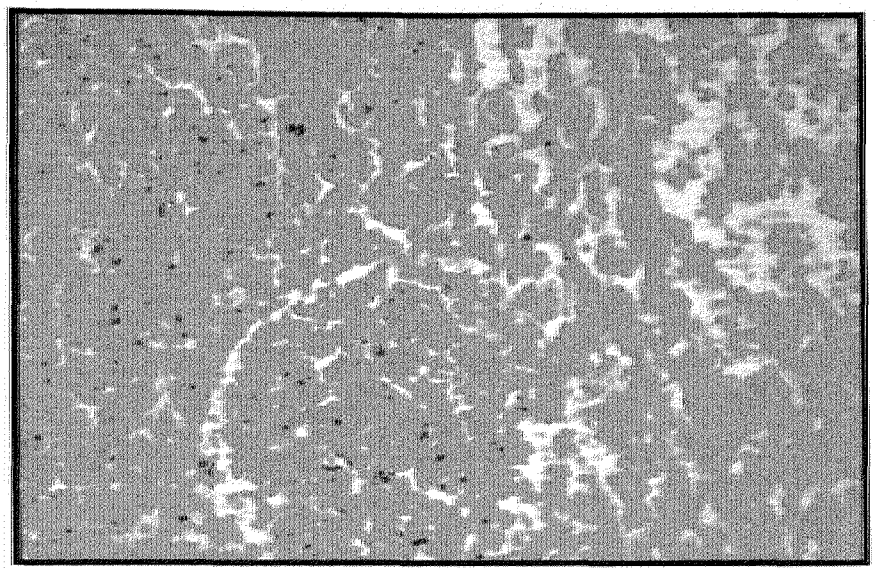

Figure 36: Microphotograph of section of spleen showing red staining of the cells of the lymphoid cells indicating the presence of RNA. Note the presence of pigment in the section. Lymphoid Leucosis (Methyl green pyronin stain, X200).

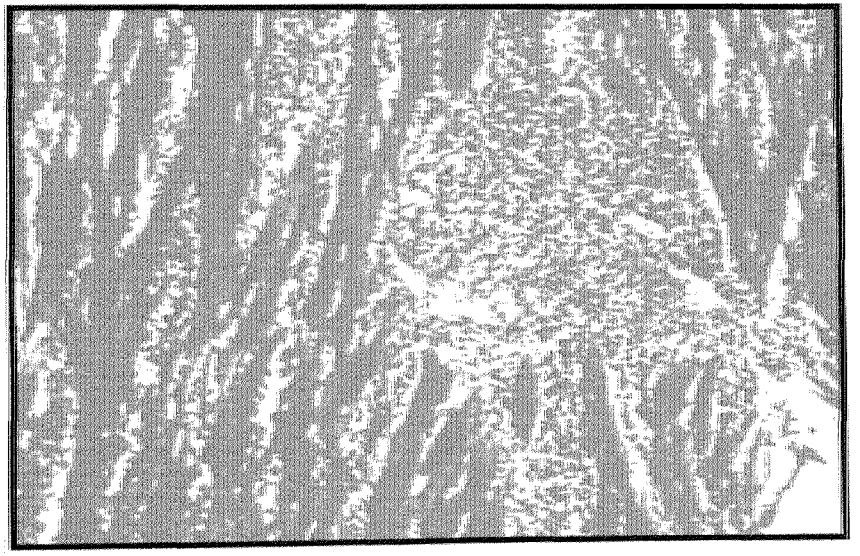

Figure 37: Microphotograph of the section of heart showing lymphoid nodule composed of uniformm sized lymphoid cells. Lymphoid Leucosis (H&E, X200).

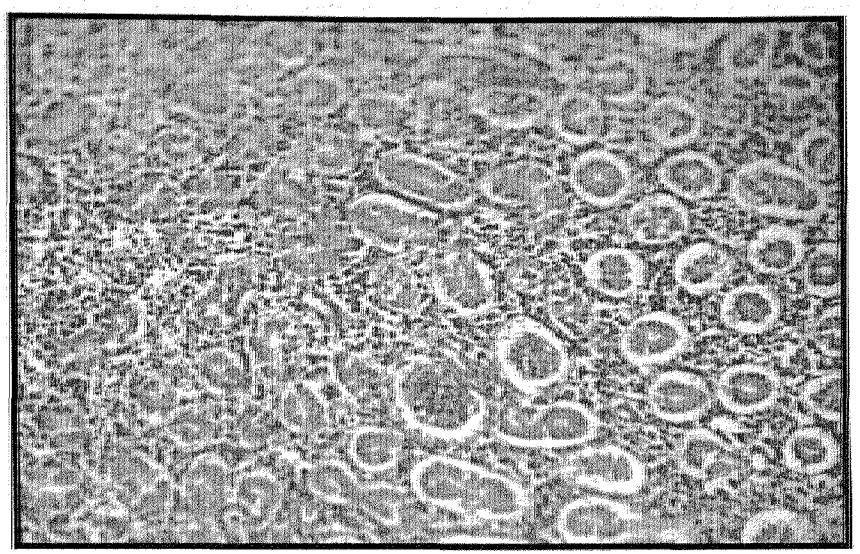

Figure 38: Microphotograph of kidney section showing diffuse infiltration of lymphoid cells in between tubules. Lymphoid Leucosis (H&E, X200).

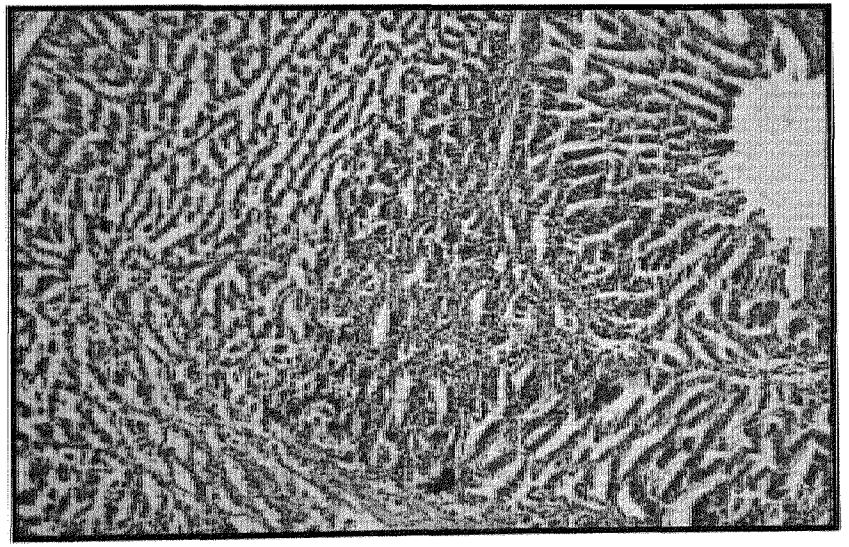

Figure 39: Microphotograph of proventriculus showing infiltration of lymphoid cells in between the glandular acini. Lymphoid Leucosis (H&E, X200).

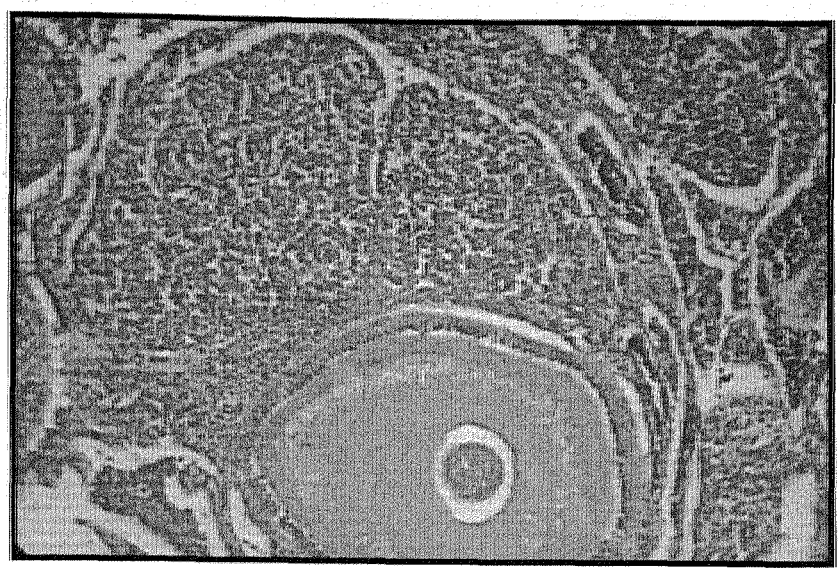

Figure 40: Microphotograph of ovary showing infiltration of uniform sized lymphoid cells in and around ovarian follicle. Lymphoid Leucosis (H&E, X200).

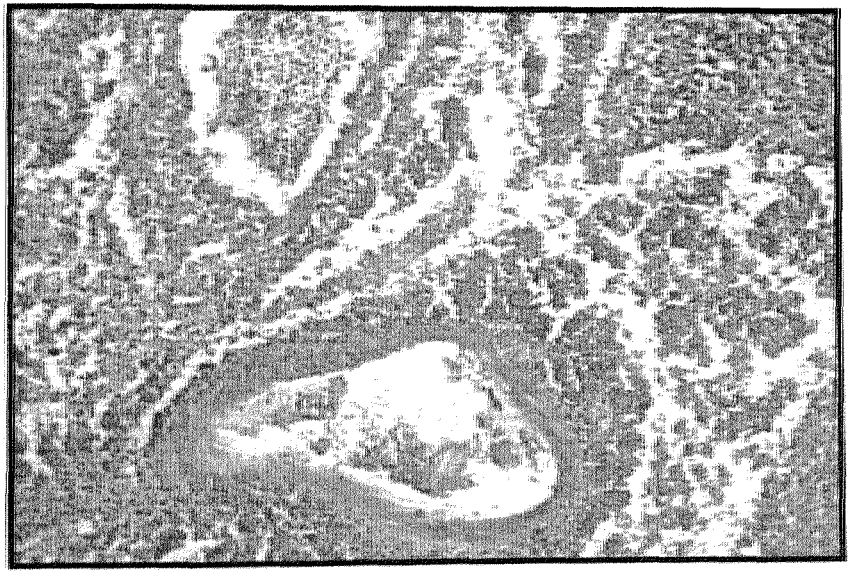

Figure 41: Microphotograph of section of lung showing heavy infiltration of uniform sized lymphoblasts almost replacing the lung tissue. Lymphoid Leucosis (H&E, X200).

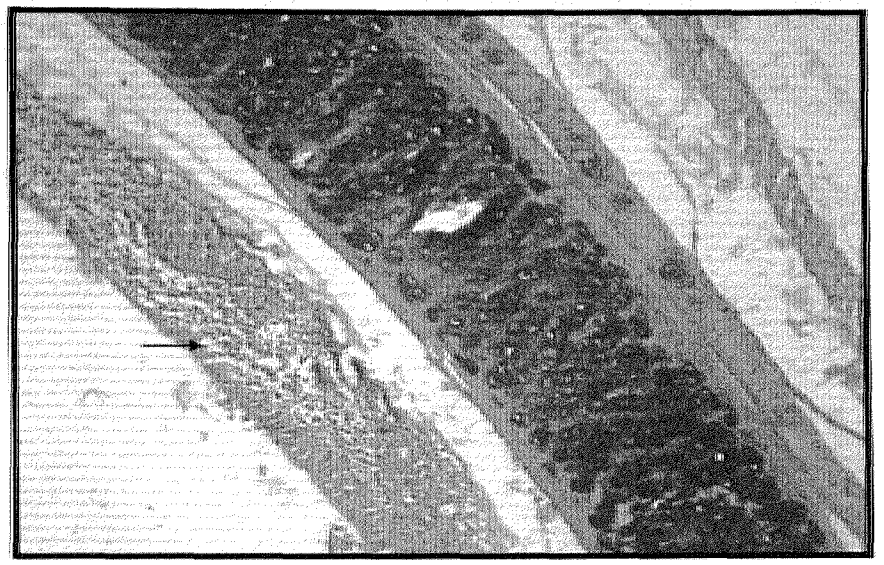

Figure 42: Microphotograph of the section of trachea showing infiltration of uniform shaped lymphoid cells in the mucosal layer (arrow). Lymphoid Leucosis (H&E, X200).

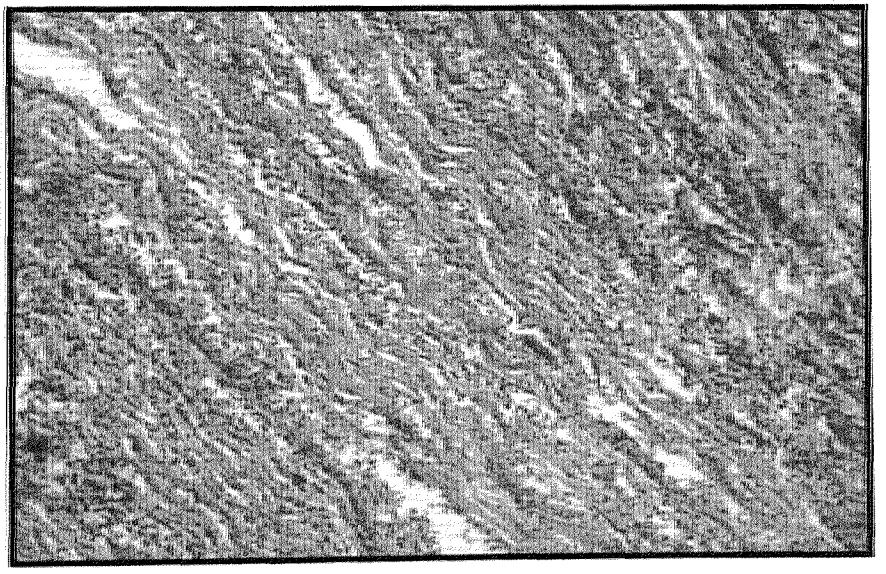

Figure 43: Microphotograph of the section of growth collected from leg region showing lymphoid cell infiltration and extensive fibrous tissue proliferation. Lymphoid Leucosis (H&E, X200).

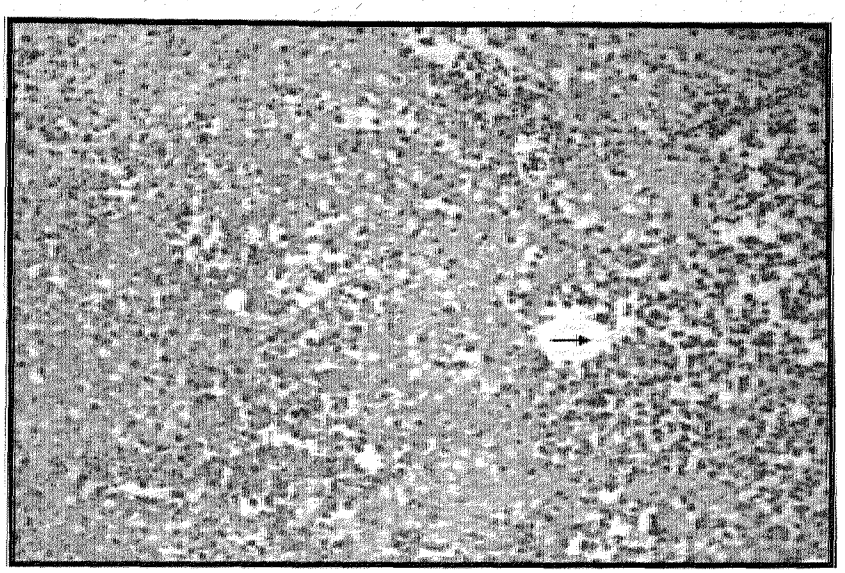

Figure 44: Microphotograph of growth collected from the junction between proventriculus and duodenum showing large, round uniform lymphoid cells with considerable amount moderate amount of fibrous tissue. Lymphoid leucosis (H&E, X200).

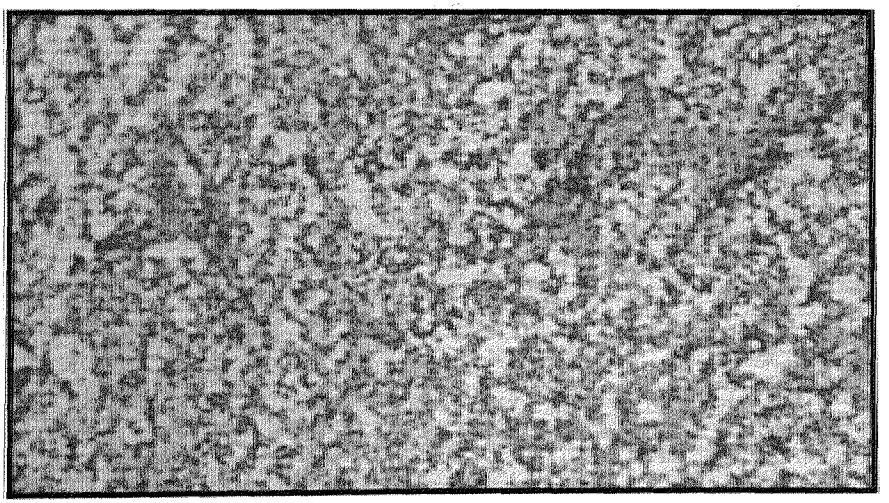

Figure 45: Microphotograph of growth collected from the junction between proventriculus and duodenum showing uniform sized lymphoid cells. Lymphoid Leucosis (H&E, X200).

The ovarian tissue was infiltrated or even replaced by uniform sized lymphoblasts. In advanced cases the ovarian parenchyma was invariably replaced by neoplastic cells, leaving behind hardly any intact ovarian follicle and stromal tissue (fig 40). The lymphoid cells were arranged in the form of solid sheets and cords.

In lung sections heavy infiltration of uniform sized lymphoblasts almost replacing the lung tissue was observed (fig 41).

In the mucosal layer of tracheal sections diffuse infiltration of uniform sized lymphoid cells whose morphology was similar to those explained above was observed (fig 42).

The growth collected from leg region revealed large, round lymphoid cells with thin cytoplasm and clear vesicular nucleus. They were arranged in cords and sheets. Extensive fibrous tissue proliferation was noted and was confirmed as leukotic tumour depending on the uniformity in the size of lymphoid cell population (fig 43).

Microscopical examination of another growth collected from the junction between proventriculus and duodenum revealed the similar type of changes as above and was confirmed as lymphoid leucosis (fig 44, 45).

Haemangioma:

The haemangioma suspected sections consisted of numerous dilated spaces of varying size and shape distributed over lobules of the liver and ovary sections. These spaces were lined by thin endothelial cells with scanty cytoplasm and moderately large nucleus and were filled with the blood elements (fig 46). Few areas of red blood corpuscles were noted and in few areas of pinkish hyalinized mass was observed. The arrangement of the hepatic cells in cords disrupted and distorted and

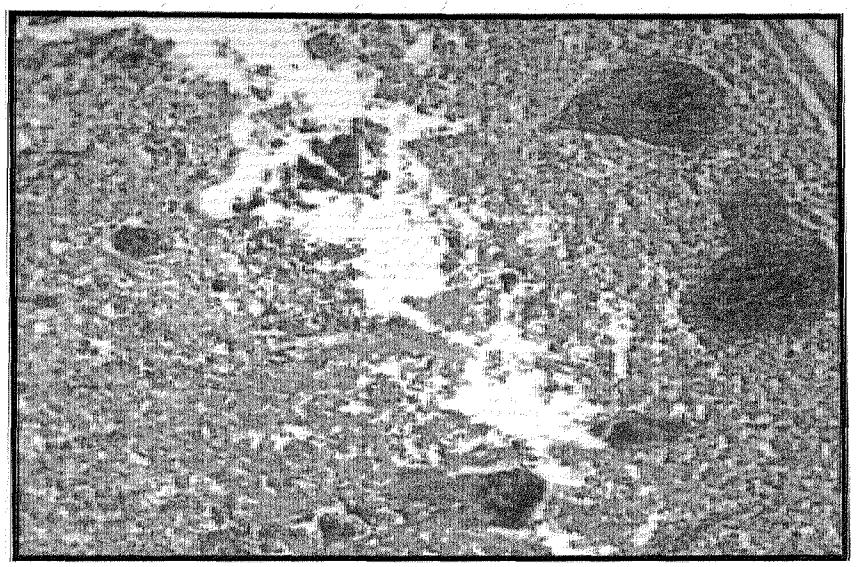

Figure 46: Microphotograph of the section of liver showing blood pockets in the parenchyma. Haemangioma (H&E, X200).

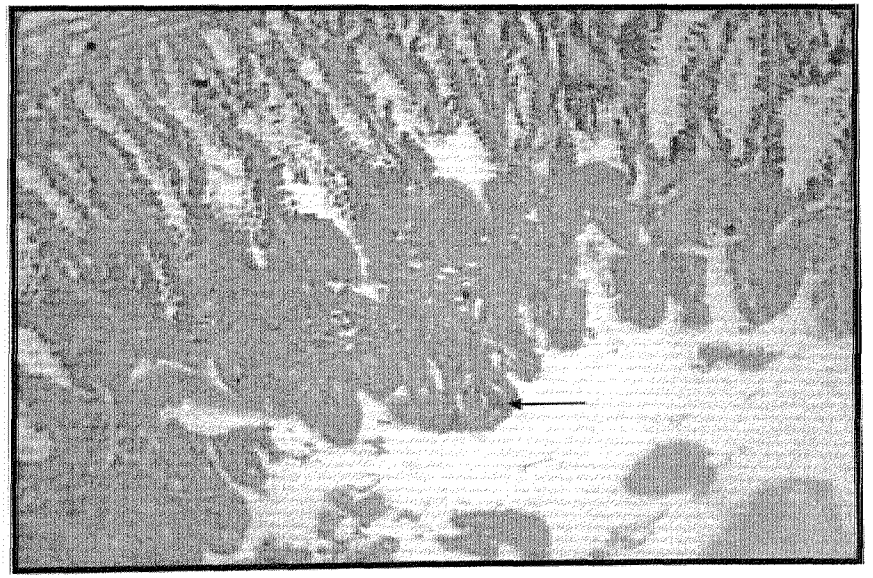

Figure 47: Microphotograph of the proventriculus: glandular acini were lined with more than one layer of epithelial cells and were thrown into papillary projections (arrow). Proventricular adenoma (H&E, X200).

the hepatic cells showed varied degrees of nuclear and parenchymatous degenerative changes.

Adenoma:

The histologic picture of three enlarged proventriculi resembled the features of adenoma. There was proliferation of glandular epithelium. Glandular acini were lined with more than one layer of epithelial cells and were thrown into folds (fig 47) and the cells lining the acinar structures were large with hyper chromatic nucleus. Moderate infiltration of lymphoid cells was observed along with the neoplastic changes.

Hepatoma:

The growths on livers histologically confirmed as hepatoma and revealed numerous proliferating hepatic cells arranged in groups with a tendency towards formation of acini like structures with discernable scanty stroma (fig 48). The hepatic cells were very much enlarged with granular cytoplasm, more vesicular nuclei and prominent nucleoli. Degeneration and necrosis with pyknotic, karyolytic nuclear changes were noted in the sections.

Nephroblastoma:

Histologically, the growths on kidney showed ill defined nests of cells with mitotic figures, lobules differentiated towards tubules and bizarre tubules in a pseudo-glomerular arrangement and was confirmed as nephroblastoma (fig 49).

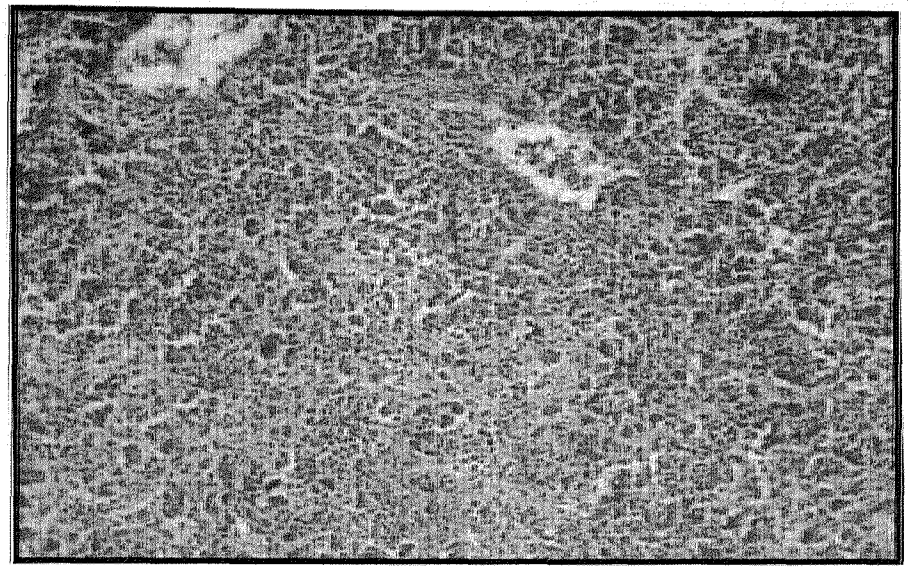

Figure 48: Microphotograph of the section of liver showing proliferating hepatic cells arranged in groups with a tendency towards formation of acini like structures (ar. Hepatoma (H&E, X200).

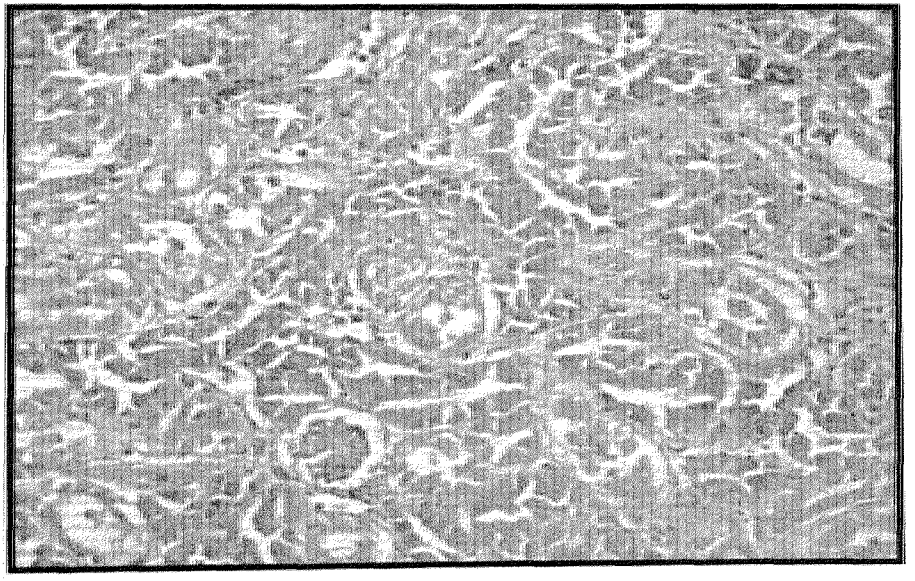

Figure 49: Microphotograph of the section of kidney showing bizarre tubules in a pseudo- glomerular arrangement. Nephroblastoma (H&E, X200).

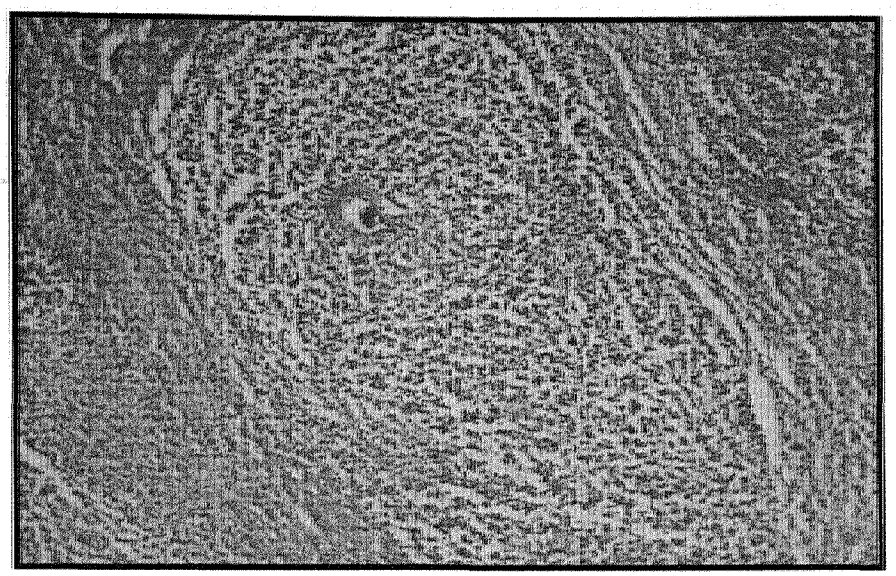

Figure 50: Microphotograph of the section of ovary showing gyriform arrangement of mature granulosa cells limited by fibrous cords. Ovarian granulosa cell tumour (H&E, X200).

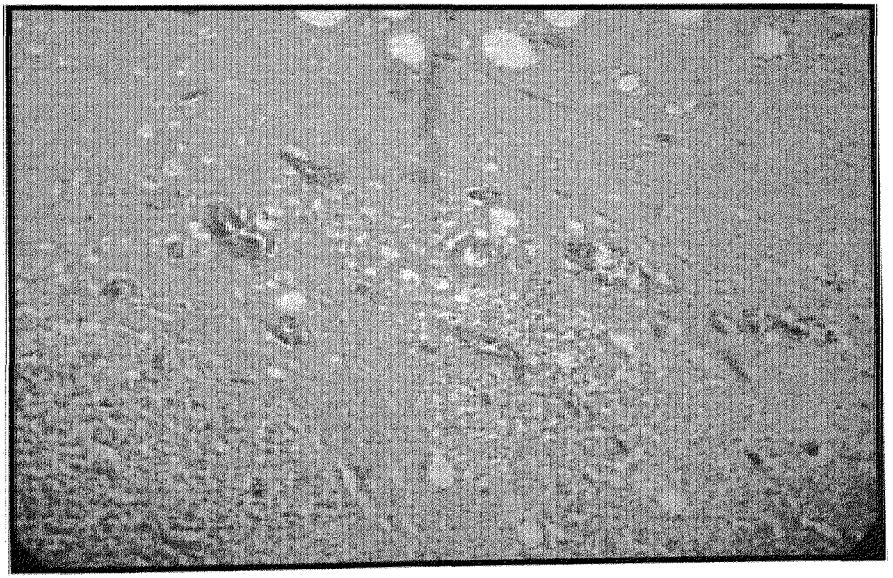

Figure 51: Microphotograph of growth showing thick, irregular bony trabaculae with osteoblasts interminigled with considerable amount of fibrous tissue. Osteofibroma (H&E, X200).

Ovarian granulosa cell tumour:

Histologically, mature granulosa cells interspersed with delicate blood vessels and a quantity of filamentous connective tissue strands replaced the normal parenchyma of ovary. Small cavities, surrounded by the solid masses or cords of mature and immature granulosa cells with concentrically arranged fibrous connective tissue, supported the formation was observed with large numbers of elaborate gyriform arrangements (fig 50).

Fibroma:

The sections of growths microscopically consisted of predominant and varying numbers of fibroblasts with spindle shaped nuclei. The fibroblasts and the collagen were arranged in parallel interwining and anastomosing fashion with small clumps or nests of cells. In few areas infiltration of lymphocytes in between the fibrous strands was noted.

Osteofibroma:

The section of growth collected from gizzard region consisted of well differentiated, thick, irregular bony trabaculae with osteoblasts intermingled with considerable amount of fibrous tissue and was confirmed as chondroma (fig 51).

Chondroma:

Histologically, the section of growth collected from skin of head region revealed round or ovoid cells set in a bluish matrix and were arranged singly (fig 52). Considerable amount of fibrous tissue and was confirmed as osteofibroma.

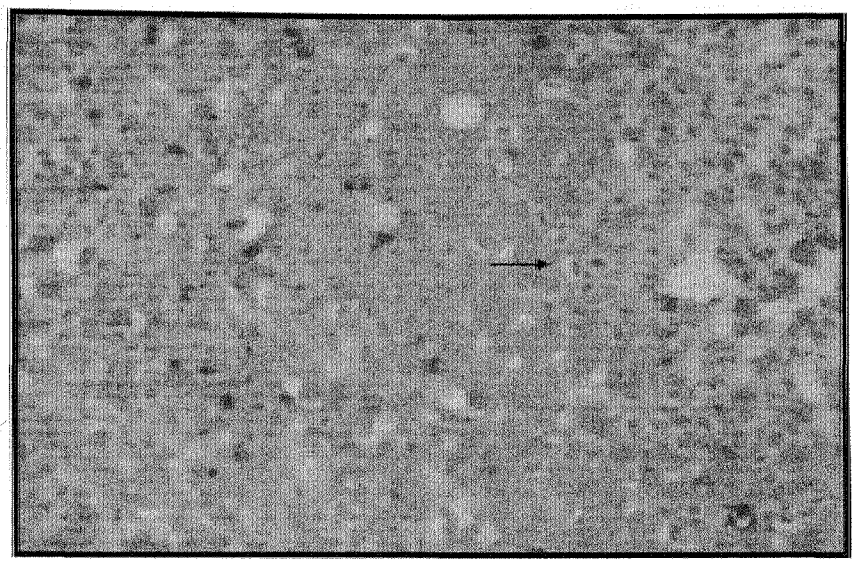

Figure 52: Microphotograph of the section of growth showing lacunae with single round to ovoid chondrocyte in them (arrow). Moderate amount of fibrous tissue can be discerned. Chondroma (X400).

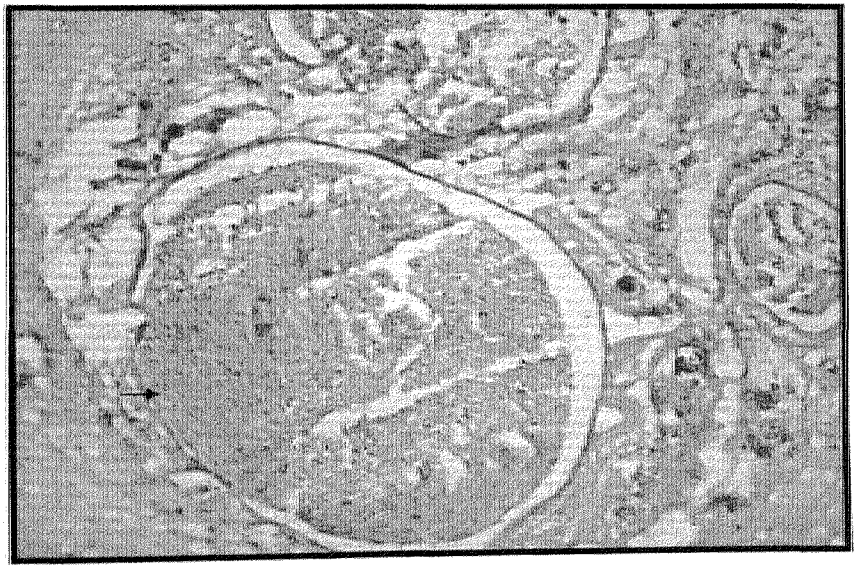

Figure 53: Microphotograph of the section showing proliferation of endothelium of the blood vessel. Endothelioma (H&E, X200).

Endothelioma:

The sections of spleen microscopically consisted of proliferating endothelial cells into a dense mass in the blood vessel. Occlusion of the vessel leaving mere clefts for blood channels due to inward growing spindle cells from the blood vessel and was confirmed as endothelioma (fig 53).

4.5 IMMUNOHISTOCHEMICAL STUDIES

A total of 29 samples were subjected to immunohistochemical staining technique. The stained tumour sections demonstrated PCNA labelling in the tumour tissue and demonstrated different levels of staining intensity and indicated the activity of the cells. Pink to red stained nuclei (2+) of lymphoid cells present between the cells of various organs indicated positive for LL (fig 54, 55).

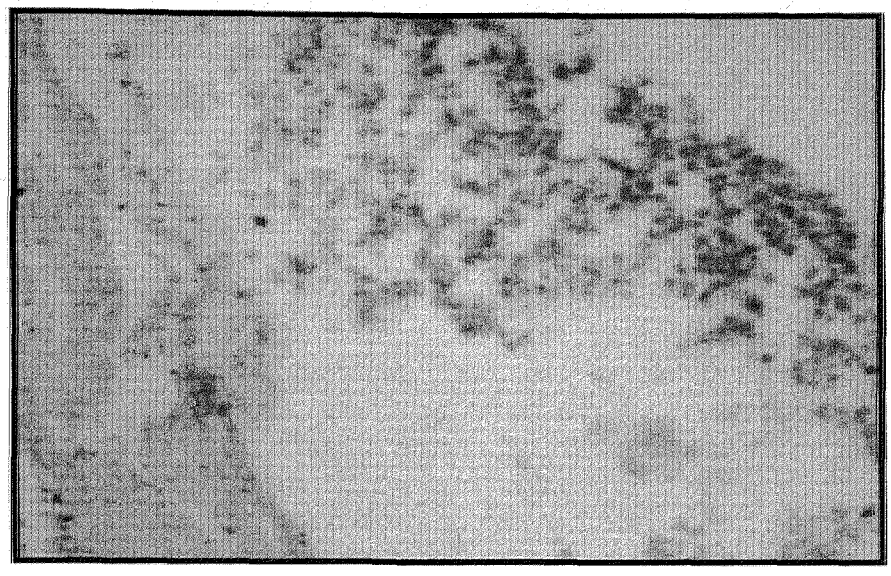

Figure 5.4: IHC stained microphotograph of section of heart showing red stained lymphoid cells positive for PCNA. (IHC staining counter stained with Mayer's Haematoxylin, X200).

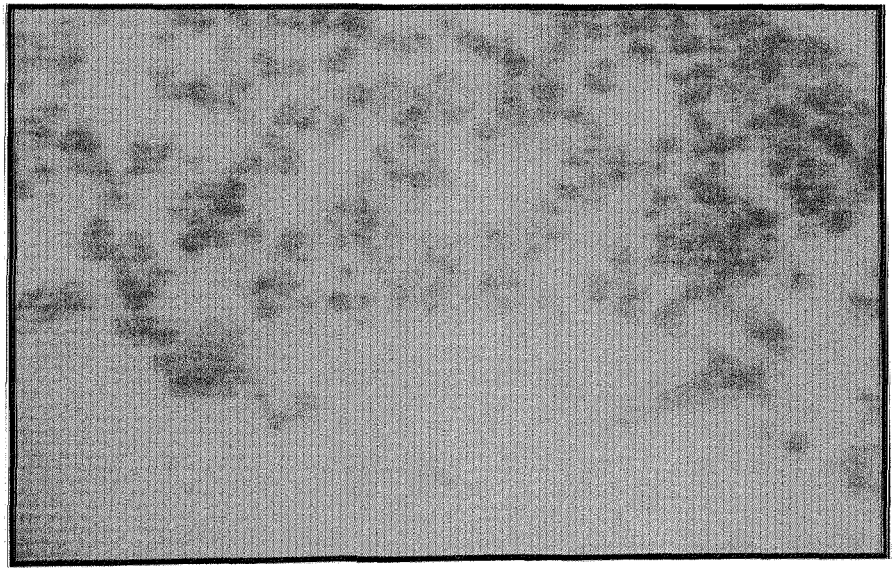

Figure 5.5: IHC stained microphotograph of section of heart showing red stained lymphoid cells positive for PCNA. (IHC staining counter stained with Mayer's Haematoxylin, X400).

DISCUSSION

CHAPTER 5

Discussion

Study on the incidence of various poultry tumours was conducted by collecting the samples from suspected cases at necropsy in the department of Pathology, Project Directorate on Poultry, Hyderabad, experimental cases in department of Microbiology and private farms of Hyderabad. A total of 72 cases and 189 samples were accumulated and were subjected to various diagnostic methods. The results obtained through various diagnostic techniques were compiled and discussed.

5.1 INCIDENCE

The incidence of tumours was recorded as highest (46.93%) in birds ranging from 8 to 18 weeks of age and it was the least (4%) in birds of age group less than 8 weeks. These findings were in agreement with the studies of Deka and Grewal (1982). However, in their study, they have not included leukotic tumours as Marek's disease and was recorded as the most frequent tumour in the age group of 8 to 18 weeks and the overall tumour incidence was recorded highest in the respective age group.

The highest incidence of tumours was recorded in Rajasree breed (46%) followed by Rajasree crossbreeds (18%), White Leghorns (12%), Aseel (10%), White Leghorn crossbreeds (6%) and Vanaraja (6%) and it was recorded least in Kadaknath breed (2%). The highest incidence of tumours in Rajasree and its high yielding strains

might be due to the fact that the bulk of the population under present study was found to be the above breed.

The incidence of tumours was high in female birds (87.5%) when compared with males (12.5%). These findings were in accordance with the results of Ahmed and Sastry (1972), Deka and Grewal (1982) and Talebi *et al.* (1993).

On the basis of type of tumours, the highest incidence was recorded for Marek's disease (43.05%) followed by Lymphoid leucosis (27.77%), other leukotic tumours (8.33%), hepatoma (4.16%), adenoma (4.16%), fibroma (2.77%), haemangioma (2.77%) and least (1.38%) for granulosa cell tumour, nephroblastoma, osteofibroma, endothelioma and chondroma. The highest incidence of Marek's disease was in agreement with the studies of Iyer (1960), Ajinkya and Sardeshpande (1969) and Reece (1996).

Three cases of hepatoma, three cases of proventricular adenoma, two cases of fibroma, two cases of haemangioma and each one case of granulosa cell tumour, nephroblastoma, osteofibroma, endothelioma and chondroma were recorded in the present study.

The incidence of hepatoma in chickens was reported by Olson and Bullis (1942), Christopher *et al.* (1968) and Ahmed and Sastry (1972). Two cases of proventricular adenoma in domestic fowl were reported by Reece (1996). Lower incidence of fibromas in chickens was reported by Singh and Singh (1968); Ajinkya and Sardeshpandey (1969); Ahmed and Sastry (1972); Brar and Grewal (1989); Reece (1992) and Reece (1996). Higher incidence of haemangiomas and haemangiosarcomas in broiler chicken were reported by Campbell and Appleby (1966) and Hemsley (1966). Reports on

incidence of haemangiomas in livers of poultry were recorded by Iyer (1960); Christopher and Rao (1964); Awadhiya and Jain (1967); Ahmed and Sastry (1972) and Reece (1996). The lower incidence of nephroblastoma was in agreement with the findings of Ahmed and Sastry (1972). Osteogenic sarcomas have been reported in a few chickens (Reece, 1996) and also in a number of other avian species (Reece, 1992). The rare incidence of ovarian granulosa cell tumour was reported by Shukla and Iyer (1960); Sharma and Singh (1968) and Ahmed and Sastry (1972); Rao and Chevudary (1981) and Reece (1992) supports the rare occurrence or incidence of chondromas in birds.

5.2 CYTOLOGICAL STUDIES

Sixty one (67.78%) of the smears were diagnosed as Marek's disease depending on the marked pleomorphism of the lymphoid cells observed. These findings were in the agreement with the report of Biggs (1973).

In the present study, 23.33% of the smears were diagnosed as lymphoid leucosis as the features of lymphoid cells were with thin rim of finely granular cytoplasm and vesicular nucleus and uniformity in size and appearance. These findings were supported by the reports of Olson (1942); Vinaraghavan and Nair (1965) and Soman and Sethi (1972).

5.3 MOLECULAR STUDIES

DNA of 30 (50.85%) samples were positive with MD specific primers M1 and M2 by producing an amplicon size of 302bp approximately which is characteristic of

132bp repeats of genome of MDV. These findings were in agreement with reports of Becker *et al.* (1992); Davidson *et al.* (1995) and Krol *et al.* (2007).

Twenty five (42.37%) samples were positive for ALV by using pair of primers (AD1 and H5) targeting conserved *Pol* gene sequence among these subgroups (A-D). These results coincided with the reports of Smith *et al.* (1998a) and Latif and Khalafalla (2005). The DNA of the samples was amplified with ALV specific primers and gave an amplicon of size 326bp.

The DNA from other 4 (6.78%) samples was neither amplified for MDV nor for ALV with any of the respective primers. The above samples grossly resembled the lymphomas of either MDV or ALV, and, histologically, infiltration of lymphoid cells was observed. This might be due to other lymphoproliferative diseases viz. Reticuloendotheliosis as the lesions often resemble the lesions caused by Marek's disease virus (Davidson *et al.*, 1995).

5.4 GROSS LESIONS

Of 72 cases, 25% were suspected for Marek's disease, 62.5% for Lymphoid leucosis, 9.72% for fibromas and 2.77% for haemangiomas grossly.

The gross lesions were characterized by enlargement and diffusely distributed grayish white nodules on various organs viz. liver, spleen, heart, kidney, proventriculus and ovaries though, these lesions were not specific for Marek's disease but were also observed by various workers viz. Benton and Cover (1957); Biggs (1973); Panda *et al.* (1983); Kobayashi *et al.* (1986); Ghosh *et al.* (1989); Kalyani (2006) and Balachandran *et al.* (2009) supported the present observations.

Forty five (62.5%) of samples were suspected for lymphoid leucosis grossly revealed enlargement of visceral organs and presence of multiple nodular tumours on visceral organs. These findings were in agreement with the reports of Olson (1942); Burmester and Dennington (1947); Viraraghavan and Nair (1965); Soman and Sethi (1972); Wadsworth *et al.* (1981); Reddy (1990); Ghosh *et al.* (2004) and Latif and Khalafalla (2005). The nodular, miliary and diffuse forms were coinciding with the reports of Soman and Sethi (1972) and Payne and Purchase (1994). Ovaries were grossly enlarged, had tumour masses of cauliflower like appearance and these findings were in agreement with the observations of Reddy (1990).

The growths 7 (9.72%) were suspected for fibroma were roughly round, firm and hard in consistency and on sectioning presence of fibrous tissue bundles running across were observed. These findings were in agreement with the reports of Singh and Singh (1968); Brar and Grewal (1989) and Reece (1992, 1996).

Two (4%) suspected for haemangioma revealed reddish specks on the organs. Reports of Christopher and Rao (1964); Brar and Grewal (1989); Reece (1992) and Reece (1996) were in support to the present findings.

5.5 HISTOPATHOLOGICAL STUDIES

One hundred and twenty three (65.07%) samples were histologically confirmed as Marek's disease, 55 (29.10%) as LL and 14 (7.4%) as other neoplastic conditions which included cases of adenoma, hepatoma, haemangioma, fibroma, nephroblastoma, chondroma, granulosa cell tumour, endothelioma and osteofibroma.

The lesions of Marek's disease in the present study were the lymphomas of various visceral organs. These findings were in support with the observations of Biggs *et al.* (1965), Benton and Cover (1957). The changing scenario of MD from classical form to acute form might be due to the continuation of an evolutionary shift of MDVs towards increasing virulence (Witter, 1998).

Microscopical lesions of livers suspected for the lymphomas consisted of infiltration of pleomorphic lymphoid cells including small, medium and large lymphocytes, lymphoblasts and reticulum cells, compressing and obliterating the normal structure of organ. In most of the liver sections marked degree of enlargement of vessells with presence of tumour emboli indicated metastasis to various organs and malignancy. Non pyroninophilic expression of tumour cells with methyl green pyronin stain confirmed the diagnosis. These findings were almost similar to the lesions described by Benton and Cover (1957).

In spleen sections, a clear demarcation between the normal lymphocytes and neoplastic cells was noted and were found mostly perivascular and marked thickening of the blood vessels was noted. Biggs (1973) stated that the proliferative lymphoid involvement is characteristically perivascular in Marek's disease.

Proliferative lymphoid changes were observed in kidneys and heart. Hyperplasia of lymphoid nodules and infiltration of lymphoid cells in submucosa were observed in proventriculi and these findings were in agreement with the reports of Prakash and Rajya (1970).

The lesions observed in ovaries were infiltration of pleomorphic lymphoid cells and are in accordance with the reports of Payne and Biggs (1967); Pradhan and Nayak (1972) and Reddy (1990).

The histological diagnosis of MD in the present study was made based on pleomorphism of lymphoid series and the same were described by Burmester and Witter (1966) and Biggs (1967).

The histological sections of liver revealed diffuse and focal infiltration of uniform sized lymphoid cells resulting atrophy of hepatocytes due to compression and replacement. These features were confirmation for lymphoid leucosis (Cooper *et al.*, 1968; Calnek and Adldingar, 1971). In nodular type of lymphomas, the nodules were surrounded by a band of fibroblast like cells and consisted of aggregates of uniform large lymphoid cells or lymphoblasts of early developmental stages. These findings were in coincidence with the observation of Gross *et al.* (1959). Presence of tumour emboli in the blood vessels indicated malignancy and sections revealed pyroninphilic positive lymphoblasts when stained with methyl green pyronin stain by presence of red stained RNA in the cytoplasm indicated that the cells were immature and rapidly dividing (Cooper *et al.*, 1968).

Depletion of lymphocytes in and around germinal centers and clear nodule formation by neoplastic lymphoid cells in spleen were observed and similar type of lesions were reported by Prukova *et al.* (2007) and described that depletion of lymphocytes in the thymus, bursa and spleen is an essential feature in the ALV infected birds.

Sections of heart revealed diffuse infiltration of lymphoid cells among the myofibrils, separation and rupture of muscle bundles. Similarly, Olson (1942) stated that heart and proventriculus were the sites of predilection for metastasis and diffuse infiltration of neoplastic lymphoid cells. Moderate fibrous tissue proliferation in the submucosa of the proventriculi in this study was also in agreement with the above report.

Ovary sections were characterized by invasion of normal tissue by homogenous population of lymphoblasts. These lesions were similar to the observations of Siccardi and Burmester (1970) and Reddy (1990).

Heavy infiltration of uniform lymphoid cells in lungs and kidney sections was observed and Olson (1942) reported metastases of lymphoid tumour to lungs and kidneys.

The growths at leg region and junction between proventriculus and duodenum revealed large, round lymphoid cells interspersed with considerable amount of fibrous tissue arranged in the form of bundles and uniformity of the lymphoid cell population might be due to lymphoid leucosis.

Cavernous haemangiomas were recorded in liver and ovary sections. Histologically, distended blood spaces with thin walls composed of endothelial cells and the spaces filled with blood elements were observed. These findings were in agreement with the reports of Christopher and Rao (1964); Zaki and Mohiyuddeen (1968) and Reece (1992, 1996). Fredrickson *et al.* (1964) reported that young chicks developed haemangiomas 3 weeks to 4 months after inoculating with field strains of ALV. Burmester (1947) and Fredrickson *et al.* (1964) demonstrated that most isolates or virus

strains cause haemangiomas. So, the probable reason for the incidence of haemangiomas in this study might be due to ALV infection.

The growth collected from proventriculus was suspected for adenoma and histologically revealed epithelium lining the glandular acini was thrown into folds forming papillary projections. These findings were in accordance with the findings of Reece (1996). This tumour might be attributed to ALV since infiltration of uniform lymphoid cells in the submucosa was observed.

Hepatoma suspected sections revealed numerous proliferating hepatic cells arranged in groups with a tendency towards formation of acini like structures were similarly reported by Christopher *et al.* (1968); Kolte *et al.* (1968); Zaki and Mohiyuddeen (1968); Sah *et al.* (1970) and Reece (1996).

A case of nephroblastoma was recorded and histologically revealed ill defined nests of cells, lobules differentiated towards tubules and bizarre tubules in a pseudoglomerular arrangement. These findings are in accordance with observations made by Asdrubali *et al.* (1995) and Reece (1996). It was mentioned that nephroblastomas were experimentally produced by myeloblastosis virus and also by different strains of sarcoma virus (Payne and Purchase, 1994).

Ovarian granulosa cell tumour was recorded in the present study revealed presence of mature granulosa cells interspersed with delicate blood vessels and considerable amount of filamentous connective tissue strands by replacing the normal parenchyma of ovary. These findings were in agreement with the reports of Shukla and Iyer (1960) and Reece (1996).

A case of fibroma was reported in this study revealed predominant and varying number of fibroblasts with spindle shaped nuclei along with collagen arranged in parallel interwining and anastomosing fashion and these findings were in accordance with Singh and Singh (1968); Brar and Grewal (1989) and Reece (1992, 1996).

Growth in the gizzard region microscopically revealed well differentiated, thick, irregular bony trabeculae with osteoblasts intermingled with fibrous tissue and this can be correlated as osteosarcoma as in humans osteosarcomas frequently contained a mixture of fibroblastic, myxomatous, cartilagemous and osseous tissue (Aho and Aho, 1982). Similar observations were reported by Campbell and Appleby (1966) and Reece (1992, 1996).

The histological findings of chondroma consisted of round to ovoid chondrocytes set in a bluish matrix and were arranged singly were similar to the observations made by Reece (1992) and Monen (2002).

Endothelioma histologically consisted of proliferating endothelial cells grown inward from blood vessel and resulted in occlusion of blood vessels leaving mere clefts for blood channels. These findings were in line with the reports of Furth (1933) and Mladenov *et al.* (1967). Mladenov *et al.* (1967) demonstrated the occurrence of endothelioma by experimentally inoculating MC29 strain of ALV into birds.

5.6 IMMUNOHISTOCHEMISTRY

Proliferating cell nuclear antigen is a cell cycle related nuclear protein and labeling of PCNA provides a useful evaluation of the proportions of proliferating cells in normal and neoplastic cell populations (Robbins *et al.*, 1987 and Berry *et al.*, 2006).

Elevated levels of PCNA appear in the late G1 phase and become maximal during the S phase of proliferating cells (Kurki *et al.*, 1986).

In the present study, grades up to 2+ of the stained cells were observed in the sections positive for leucosis. This indicated that the lymphoid cells were in various phases of cell cycle. Minimal (1+) nuclear staining would be consistent with G1 phase cells since PCNA synthesis begins in the late stages of this phase. The distinct and intense 2+ and 3+ nuclear staining would then identify cells in S phase where PCNA production is greatest (Foley *et al.*, 1991). As the majority of the lymphoid cells were graded under 2+, it was correlated that the lymphoid cells were in actively dividing phase. So, as the tumour cells proliferate, they displace and compress cells of the organ rather than infiltrate among them.

SUMMARY

CHAPTER 6

Summary

Neoplasms in poultry are recognised as one of the more common disease conditions of the domestic chicken. Of them Marek's disease and Avian Leucosis Complex were more important due to the significant economic losses by lowered production and mortality caused by them and their frequency of occurrence. Keeping in view of the importance of neoplasms affecting economy of the poultry industry, the present study is intended with the following objectives:

1) To know the incidence of tumours in poultry

2) To diagnose the type of tumor by various diagnostic techniques.

Samples from the birds of various age groups presented to the department for necropsy examination, samples collected from Avian Health division of Project Directorate on Poultry, Hyderabad and from other private farms in and around Hyderabad at necropsy were collected. One hundred and eighty nine samples (suspected growths and organs) from 72 birds were collected and were subjected to cytological, molecular, histopathological and immunohistochemical studies.

The incidence of tumours was recorded as highest (46.93%) in birds ranging from 8 to 18 weeks of age and it was the least (4%) in birds of age group less than 8 weeks. The highest incidence of tumours was recorded in Rajasree breed (46%) followed by Rajasree crossbreeds (18%), White Leghorns (12%), Aseel (10%), White Leghorn crossbreeds (6%) and Vanaraja (6%) and it was recorded least in the Kadaknath breed

(2%). The incidence of tumours was high in female birds (87.5%) when compared with males (12.5%).

On the basis of type of tumours, the highest incidence was recorded for Marek's disease (43.05%) followed by Lymphoid Leucosis (27.77%), other leukotic tumours (8.33%), hepatoma (4.16%), adenoma (4.16%), fibroma (2.77%), haemangioma (2.77%) and least (1.38%) for granulosa cell tumour, nephroblastoma, osteofibroma, endothelioma and chondroma.

The collected organ or tissue impression smears (90) from 46 birds were stained with Leishman's or Giemsa stain and confirmed 61 (67.78%) smears as Marek's disease depending on the pleomorphism of lymphoid cell population and 21 (23.33%) smears as lymphoid leucosis based on the uniformity of size of the lymphoid cells. 8 (8.88%) smears were negative for either MD or LL. Cytology can give a necessary information regarding tumours based on which ready diagnosis can be made.

Fifty nine tissue samples suspected for Marek's disease and Lymphoid Leucosis were subjected for molecular diagnosis through PCR by using two sets of primers that were specific for MDV and ALV. Thirty (50.85%) samples DNA got amplified with M1 and M2 primers specific for MDV and were positive for Marek's disease. 25 (42.37%) samples DNA was amplified with AD1 and H5 primers specific for *Pol* gene of ALV and were positive for ALV infection. Remaining 4 (6.78%) samples DNA was neither amplified for MDV nor for ALV with any of the above primers. The use of PCR was limited to the diagnosis of tumours of viral origin only. It is the quick method to diagnose MD and ALV infections.

Grossly, of 72 suspected tumour cases, 18 (25%) were suspected as Marek's disease, 45 (62%) as Lymphoid Leucosis, 2 (2.77%) as haemangiomas and 7 (9.72%) as fibroma.

Cases suspected for Marek's disease were characterized by gross enlargement and diffusely distributed grayish white nodules on various organs viz. liver, spleen, heart, kidney, proventriculus and ovaries. Forty five (62.5%) samples suspected for lymphoid leucosis grossly revealed enlargement of visceral organs and presence of multiple nodular tumours on them. The nodular, miliary and diffuse forms were specifically observed in suspected liver samples. Ovaries were grossly enlarged and had tumour masses of cauliflower like appearance.

Seven growths (9.72%) suspected for fibroma were roughly round, firm and hard in consistency and on sectioning presence of fibrous tissue bundles running across were observed. Two (4%) suspected for haemangioma revealed reddish specks on the organs. Gross examination of tumours was found to be an aid in diagnosing the type of tumours in poultry.

One hundred and twenty three (65.07%) samples were histologically confirmed as Marek's disease, 55 (29.10%) as LL and 14 (7.4%) as other neoplastic conditions that included cases of adenoma (3), hepatoma (3), haemangioma (2), fibroma (2), nephroblastoma (1), chondroma (1), granulosa cell tumour (1), endothelioma (1) and osteofibroma (1).

The histologicall diagnosis of MD in the present study was made on the basis pleomorphism of lymphoid series. Sections of livers consisted of infilltration of pleomorphic lymphoid cells including small, medium and large lymphocytes,

lymphoblasts and reticulum cells, compressing and obliterating the normal structure of organ. In spleen sections, a clear demarcation between the normal lymphocytes and neoplastic cells was noted and were found to be perivascular mostly and marked thickening of the blood vessels was noted. Proliferative lymphoid changes were observed in kidneys and heart. Hyperplasia of lymphoid nodules and infiltration of lymphoid cells in submucosa were observed in sections proventriculi. Ovary exhibited extreme infiltration of lymphocytes displacing the entire ovarian tissue and only a few scattered follicles were discerned.

Histologically 20 cases (27.77%) were diagnosed as lymphoid leucosis. Extensive proliferation and infiltration of lymphoid cells were recorded in liver sections. The histological pattern of livers that were nodular type revealed, nodules that were usually surrounded by a band of fibroblast like cells and consisted of aggregates of uniform sized large lymphoid cells or lymphoblasts of early developmental stage. Sections of spleen revealed clear lymphoid nodule formation, periarteriolar nodules composed of large lymphoblasts with clear vesicular nucleus and in a few sections, the lymphoid transformation in the periphery, underneath the capsule and depletion of lymphocytes in and around germinal centres and thickened blood vessels were observed. Diffuse accumulation and proliferation of lymphoid cells in sections of heart, lung, kidney, submucosa of proventriculus, mucosal layer of trachea and ovaries were observed. The growths at leg region and junction between proventriculus and duodenum revealed large, round lymphoid cells interspersed with extensive proliferation of fibrous tissue arranged in the form of bundles.

Cavernous haemangiomas were recorded in liver and ovary sections. Histologically, distended blood spaces with thin walls composed of endothelial cells and the spaces filled with blood elements were observed. The probable reason for the incidence of haemangiomas might be due to ALV infection.

The histological picture of three enlarged proventriculi resembled the features of adenoma (4.16%). There was proliferation of glandular epithelium. Glandular acini were lined with more than one layer of epithelial cells and were thrown into folds forming papillary projections.

Hepatoma (4.16%) suspected sections revealed numerous proliferating hepatic cells arranged in groups with a tendency towards formation of acini like structures. The hepatic cells were very much enlarged with granular cytoplasm, more vesicular nuclei and prominent nucleoli.

A case of nephroblastoma (1.38%) was recorded and histologically revealed ill defined nests of cells with mitotic figures, lobules differentiated towards tubules and bizarre tubules in a pseudo- glomerular arrangement.

Ovarian granulosa cell tumour (1.38%) was recorded in the present study revealed presence of mature granulosa cells interspersed with delicate blood vessels and considerable amount of filamentous connective tissue strands by replacing the normal parenchyma of ovary.

A case of fibroma (1.38%) was reported in this study revealed predominant and varying number of fibroblasts with spindle shaped nuclei along with collagen arranged in parallel interwining and anastomosing fashion.

Growth in the gizzard region microscopically revealed well differentiated, thick, irregular bony trabaculae with osteoblasts intermingled with fibrous tissue and this can be correlated as osteosarcoma or osteofibroma (1.38%).

Histologically the section of growth collected from skin of head region revealed round or ovoid cells set in a bluish matrix and were arranged singly and was confirmed as chondroma (1.38%).

The sections of spleen revealed proliferating endothelial cells into a dense mass in the blood vessel. Occlusion of the vessel leaving mere clefts for blood channels due to inward growing spindle cells from the blood vessel was noted and was confirmed as endothelioma (1.38%). Histopathological studies are the best and reliable method in diagnosing the type of tumour.

A total of 29 samples were subjected to immunohistochemical staining technique and the stained sections demonstrated PCNA labelling in the tumour tissue and demonstrated different levels of staining intensity and indicated the activity of the cells. Pink to red stained nuclei (2+) of lymphoid cells present between the cells of the organs were positive for LL. As the majority of the lymphoid cells were graded under 2+, it was correlated that the lymphoid cells were in actively dividing phase. PCNA staining provides a useful evaluation of the proportions of proliferating cells in normal and neoplastic cell populations.

From the above studies it can be concluded as:

1) Incidence of tumours was more between 8-18 weeks age group and females were more affected.

2) The incidence of Marek's disease was found to be more followed by Lymphoid leucosis.

3) Gross examination of tumours was found to be an aid in diagnosing the type of tumours in poultry.

4) Cytology can give a necessary information regarding tumours based on which ready diagnosis can be made.

5) Histopathological studies are the best and reliable method in diagnosing the type of tumour.

6) PCR is the quick method to diagnose MD and ALV infections.

7) PCNA staining provides a useful evaluation of the proportions of proliferating cells in normal and neoplastic cell populations.

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ANNEXURE

Annexure

10X TBE buffer:

Tris base	-	54g
Boric acid	-	27.5g
EDTA	-	4.15g
Distilled water	-	500ml

1X TBE buffer

10X TBE buffer	-	10ml
Distilled water	-	90ml

0.1M PBS (pH 7.4) used for immunohistochemistry

Disodium hydrogen phosphate	-	5.9165g
Sodium dihydrogen phosphate	-	1g
Double distilled water	-	500ml
EDTA	-	0.25g
Tween 20	-	250 µl
Sodium azide	-	0.5g
Bovine serum albumin	-	5g
		500ml