

Nucleolar Organizer Region Count, PCNA and Ki-67 Indices are Diagnostic Markers of Malignancy and Cell Proliferation Rate in Bovine Lymphosarcoma

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Abstract

Lymphoid tumors comprise one of the most common groups of tumors in domestic animals. Lymphosarcoma is expressed as a specific multicentric form in calves upto six months of age (sporadic) and in adult cattle (enzootic); and as thymic form in young cattle. Eight calves of both sexes aged below six months were necropsied in the department of veterinary Pathology, college of Veterinary Science, Tirupati. During necropsy impression smears, represented tissue samples were collected for Cytopathology, histopathology, AgNOR count and immunohistochemical studies. Cytological smears revealed lymphocytes, polymorphocytes and lymphoblasts with anaplastic changes. Histopathologically, partial to complete obliteration of architecture due to the replacement of normal tissue by lymphoblastic cells in lymphnodes and other visceral organs. The mean AgNOR count of the lymphosarcoma was 4.25 and it appear as a round to irregular, dark brown to black dots of varying sizes in the nuclei. Positive expression of PCNA and Ki67 was noticed in lymphnode, liver, kidney and lung was observed. The labeling index of PCNA and Ki-67 expression and distribution of immunopositive cells may be helpful in the early diagnosis and prognosis of malignant tumors. Hence, AgNOR, PCNA and Ki-67 indices can be used as reliable markers of cell proliferation and malignancy.

Keywords: Lymphoid tumors; Enzootic bovine leucosis; Histopathology

Introduction

Bovine lymphosarcoma or Enzootic Bovine leucosis (EBL) is an exogenous, B lymphotropic retrovirus, belonging to the family Retroviridae and comprise one of the most common groups of tumors in domestic animals. Anatomically lymphoid tumors are classified as multicentric, thymic, alimentary, skin and solitary forms. Bovine lymphosarcoma is expressed as a specific multicentric form in adult cattle (enzootic bovine leukosis- EBL) and calves upto six months of age (sporadic bovine leukosis-SBL) and as thymic form in young cattle [1]. The disease is transmitted either directly by transmission of lymphocytes via blood, semen, or milk, or indirectly via insects. The disease does not spread rapidly, but in infected herds the number of seropositive animals may be 80% [2].

Although animals can become infected with BLV at any age, tumours (lymphosarcomas) are seen typically in animals over 3 years of age. Infections are usually subclinical; only 30–70% of infected cattle develop persistent lymphocytosis, and 0.1%–10% of the infected animals develop tumors. Most BLV infected cattle do not show any clinical symptoms. Some infected cattle may show nonspecific clinical symptoms such as weakness and emaciation and later, signs will depend on the site of the tumors and may include digestive disturbances, inappetance, weight loss, weakness or general debility and sometimes neurological manifestations. Superficial lymph nodes may be obviously enlarged and may be palpable under the skin and by rectal examination [3]. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells. Organs most

frequently involved are the abomasum, right auricle of the heart, spleen, intestine, liver, kidney, omasum, lung, and uterus [4].

Once infected, cattle remain infected, showing a serological response a few weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear [5]. Since there is no vaccination, the presence of antibodies is an accurate indicator of natural infection. So, eradication and control of the disease is exclusively based on screening for antibodies and segregation of serologically positive animals from negative animals. Several authors have shown that it is possible to establish BLV-free herds by identifying seropositive animals and eliminating them from the herds [6-8].

Materials and Methods

Samples collected from six male calves and two female Holstein Friesian calves below six months of age animals necropsied in the Department of Veterinary Pathology, College of Veterinary Science, Tirupati. For cytological examination, during necropsy impression smears from different organs were collected and they were stained with Leishman's stain [9].

Histopathology

Representative tissue samples from different organs were collected and fixed in 10 percent neutral buffered formalin and processed routinely for histopathological examination. Sections of 4-6 μ thickness were made and stained with Haematoxylin and Eosin [10,11].

AgNOR staining

AgNOR staining was performed as described by Krishnamurthi and Paliwal [12]. The sections were deparaffinized in xylene and hydrated through decreasing grades of ethanol to double distilled deionized water. The sections were then reacted with freshly prepared silver colloidal solution (1 part by volume of 2% gelatin in 1% formic acid and two parts by volume of 50% aqueous silver nitrate solution) in darkness for 35 min at room temperature. The silver colloidal solution was washed with double distilled ionized water. The sections were then treated with 5% sodium thiosulphate for 5 minutes and washed in double distilled deionized water, dehydrated through increasing grades of alcohol, cleared in xylene and mounted in DPX.

AgNOR counting

The number of AgNORs present in each nucleus was counted in 100 non overlapping nuclei by using a 100x oil immersion lens. At this magnification, AgNORs are visible both within and outside the nuclei.

Immunohistochemistry

Immunohistochemistry was performed using Universal Dakocytomation LSAB[®] kit to evaluate the expression of proliferative markers namely PCNA and Ki-67 in lymphosarcoma. Sections of 5-6 μ thickness were mounted on to APES (Amino Propyl EthoxySialine) coated slides and incubated overnight at 37°C.

Method

Sections were deparaffinized and rehydrated. Sections were kept in citrate buffer (10 mM, pH 6) and subjected to microwave treatment for 5 cycles each for 5 minutes at 750 watts to retrieve the antigenic sites. Sections were allowed to cool down to the room temperature. Sections were rinsed in TBS (Tris Buffer Saline). One drop of hydrogen peroxide was kept over the section for 15 minutes to block unwanted antigenic sites and to quench endogenous peroxidase activity. Sections were rinsed with TBS. Primary antibody was added onto the sections and incubated for 30 minutes. Sections were rinsed with TBS. Sections were incubated with secondary link antibody for 30 minutes. Sections were rinsed with TBS. Sections were incubated with tertiary Streptavidin peroxidase for 30 minutes. Sections were rinsed with TBS. DAB (Diaminobenzidine) was used as chromogen and the sections were incubated for 10 minutes. Sections were rinsed with distilled water. Sections were counter stained with Harris haematoxylin and mounted in DPX.

Results

Lymphosarcoma was observed in eight cattle which were necropsied in the department. All the affected animals were white cattle which included six male calves below six months of age and two female Holstein Friesian calves. Grossly the prescapular and precrucial lymph nodes of calves were enlarged than their normal size (Figure 1). The enlargement and conglomeration of mesenteric lymph nodes was observed in one female calf (Figure 2). The affected organs were soft, fleshy and greyish white in color (Figure 3). On cut section, the affected lymph nodes showed bulging and a homogenous pale appearance with no demarcation between cortex and medulla (Figure 4).



Figure 1: Lymphosarcoma: Calf: Enlargement of prescapular lymphnode.

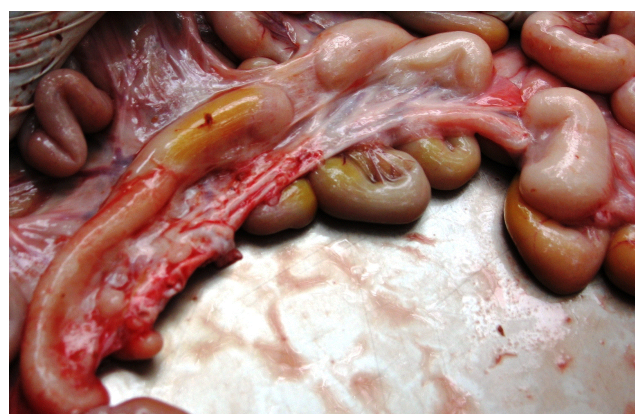


Figure 2: Lymphosarcoma: Calf: Note the enlargement and conglomeration of mesenteric lymphnodes.

Cytopathology

Impression smears collected from affected lymph nodes and organs like heart, lung, liver and spleen revealed numerous cells which included lymphocytes, prolymphocytes and lymphoblasts. The neoplastic cells were characterized by a marked variation in cell size, atypia in large cells, nuclear irregularity in smaller cells, nucleolar prominence in cells with scanty basophilic cytoplasm (Figures 5 and 6).



Figure 3: Lymphosarcoma: Calf: Note the enlargement and pale appearance of liver.



Figure 4: Lymphosarcoma: Calf: Observe the bulging and a homogenous pale appearance of prescapular lymphnode with no demarcation between cortex and medulla on cut section.

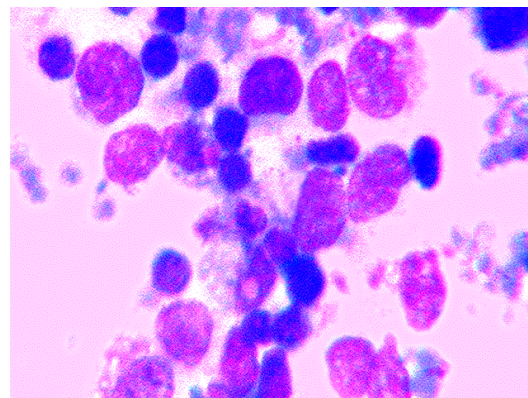


Figure 5: Lymphosarcoma: Lymphnode: Cytology: Smear showing numerous lymphocytes and lymphoblasts with anaplasia, increased nuclear to cytoplasmic ratio, hyperchromatic nucleus and 3-5 prominent nucleoli. Leishman's stain: 700X.

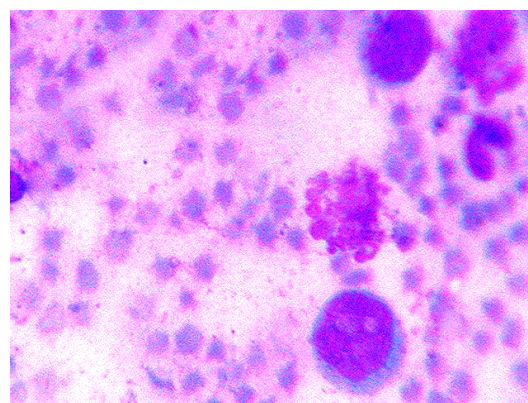


Figure 6: Lymphosarcoma: Heart: Cytology: Impression smear showing large neoplastic lymphocyte with scanty basophilic cytoplasm, prominent nucleus and nucleoli. Leishman's stain: 700X.

Histopathology

Histopathology of affected lymph nodes showed partial to complete obliteration of architecture due to the replacement of normal tissue by neoplastic cells (Figure 7). The neoplastic cells were immature lymphoblasts which were large, polyhedral with a basophilic cytoplasm and a large hyperchromatic nucleus placed eccentrically. Mitotic figures were present. Grouping of cells was observed in some areas giving the appearance of giant cells (Figures 8 and 9). Mild to moderate proliferation of neoplastic cells were seen in lung, liver and kidney (Figures 10 and 11).

AgNOR COUNTING

The AgNORs appeared as round to irregular, dark brown to black dots of varying sizes in the nuclei. The mean AgNOR count of the lymphosarcoma was 4.25 (Figure 12) and a significant correlation was found between an increased AgNOR count and histological grade.

Immunohistochemistry

Immunohistochemical studies were carried out to evaluate the expression of cell proliferation markers namely PCNA and Ki-67 in lymphosarcoma. Samples of lymphosarcoma were assessed using immunohistochemical method with monoclonal antibodies against PCNA and Ki-67.

Positive expression of PCNA was observed in 90% (7/8) of lymphosarcomacases and the protein expression was restricted to the nucleus. Positive expression of PCNA in lymphosarcoma was seen in the lymphnodes, lung, liver and kidney (Figures 13-16).

Ki-67 expression was seen in 75% (6/8) of the lymphosarcoma examined and the protein expression was restricted to nucleus. In lymphosarcoma, positive expression of Ki-67 was seen in the lymphocytes and lymphoblasts of lymphnodes, lung, liver and kidney (Figures 17-21).

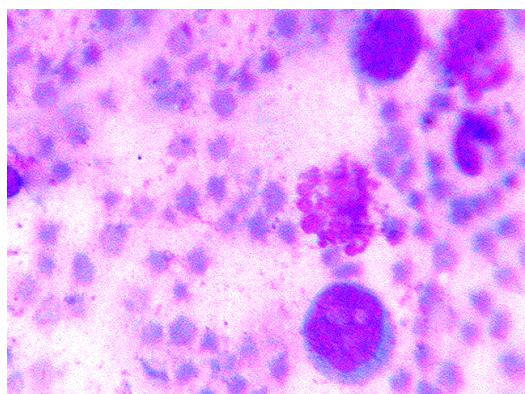


Figure 7: Lymphosarcoma: Lymph node: Note the replacement of normal architecture by proliferation of neoplastic cells. H&E: 70X.

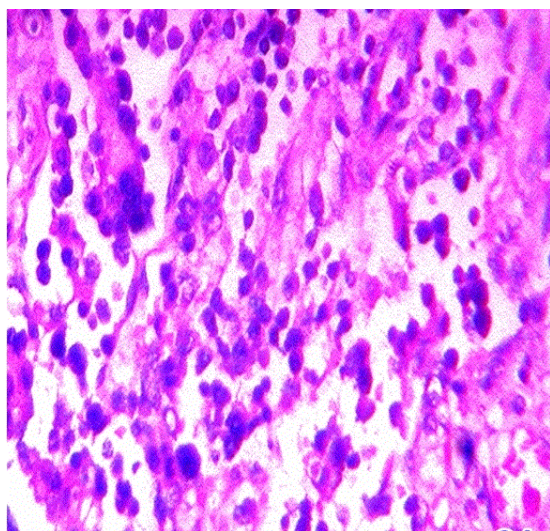


Figure 8: Lymphosarcoma: Lymph node: Section showing lymphocytes and immature lymphoblasts which were large, polyhedral with a basophilic cytoplasm and a large hyperchromatic nucleus placed eccentrically. H&E: 280X.

Discussion

In the present study, lymphosarcoma constituted 14.04% of the bovine tumors which was in contrary to the results of Plummer [13] who mentioned it as 32%. This difference in incidence might be due to the detection of affected animals only during slaughter [14]. All the affected animals were cattle. 75% of cases were seen in male calves below six months of age and the remaining 25% in adult Holstein Friesian cows. The present study revealed high incidence in male cattle (75%) than in female (25%) cattle, which was in agreement with the

reports of Singh [15,16] in buffaloes. In contrary, Yoon et al. [14] observed 100% of the lymphosarcoma cases in female cattle. In the present study, the enlargement was observed mostly in lymph nodes followed by the visceral organs, as observed by Yoon et al. [14] Sabareeswaran et al. [17] and Anusha et al. [18] reported cases of ocular lymphoma, pulmonary lymphoma, uterine lymphosarcoma respectively in buffaloes. Lymph might be the main route of metastasis to different organs as mentioned by Yoon et al. [14]. The deaths in affected cattle might be due to the complications of growing mass [4].

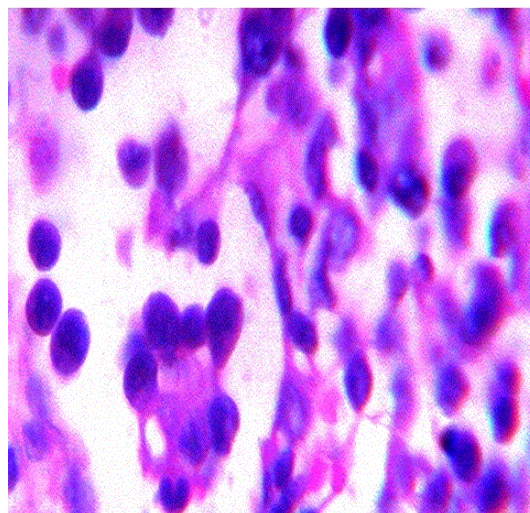


Figure 9: Lymphosarcoma: Lymph node: Note the characteristic grouping of neoplastic cells with the appearance of giant cells and with mitotic figures. H&E: 700X.

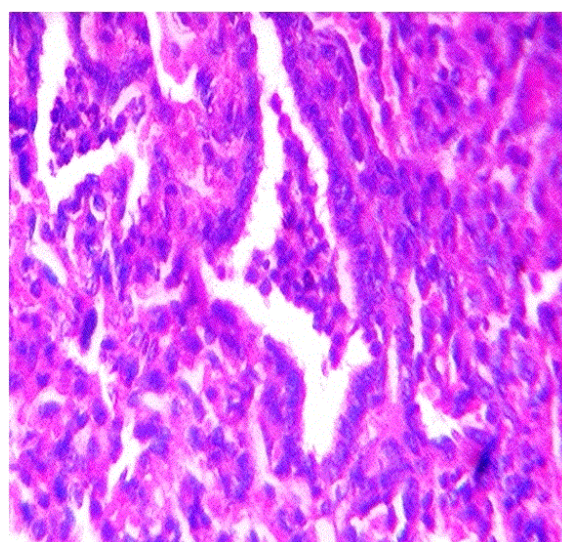


Figure 10: Lymphosarcoma: Liver: Note mild to moderate proliferation of neoplastic cells in periportal and sinusoidal spaces. H&E: 70X.

Grossly, the enlargement was noticed in prescapular and precrural lymph nodes in calves and there was enlargement and fusion of

mesenteric lymph nodes in the cow. The affected organs were soft, fleshy, greyish white and on cut section, the lymph nodes showed bulging and a homogenous pale appearance with no demarcation between cortex and medulla [14,15,19-21]. Impression smears collected from affected lymph nodes and organs like heart, lung, liver and kidney revealed numerous cells which included lymphocytes, prolymphocytes and lymphoblasts. The neoplastic cells were characterized by a marked variation in cell size, atypia in large cells, nuclear irregularity in smaller cells, and nucleolar prominence in cells with scanty, basophilic cytoplasm. Similar observations were made by Anil Kumar et al. and Sabareeswaran et al. [4,17].

Histopathology of affected lymph nodes showed partial to complete obliteration of architecture of lymph nodes due to the replacement of normal tissue by neoplastic cells. The neoplastic cells were either lymphocytes or immature lymphoblasts which were large, polyhedral with a basophilic cytoplasm and a large, hyperchromatic nucleus placed eccentrically. Mitotic figures were present. Iwama et al. [21] and Branco et al. [22] observed similar histological features.

In the present study, AgNORs appeared as round to irregular, dark brown to black dots of varying sizes in the nuclei which was in accordance with the observations of Chandrasekhar and Lalitha [23]. Malignant tumors had numerous, smaller and irregular AgNORs, dispersed throughout the nucleus whereas benign tumors had large, round, sharply defined and few AgNOR dots confined to the nucleoli. These findings were in accordance with the findings made by Crocker et al. [24], Chandravathi et al. [25] and Veena et al. [26]. In contrary, Sandhya et al. [25] observed small, homogeneously stained, regular AgNORs in benign tumors (papillomas) and large irregular dots or bizarre clusters in malignant tumors (SqCC). Variations in the size and number of the AgNOR dots might depend on the stage of the cell cycle, the transcriptional and metabolic activity of the cell or the number of NOR-bearing chromosomes in the karyotype [27].

In the present study, immunoexpression of PCNA was greater (90%) than that of Ki-67 (50%) in epithelial tumors and lymphosarcoma, which was in accordance with the study of Reszec et al. [28]. Whereas Zhong et al. [29] observed that the expression of Ki-67 was greater than that of PCNA in prostate cancer. The expression of PCNA and Ki-67 was restricted to the nucleus [28]. A moderate, homogenous distribution of Ki-67 positive neoplastic cells in the lymphosarcoma was seen in lymphnode, lung, liver and kidney [30].

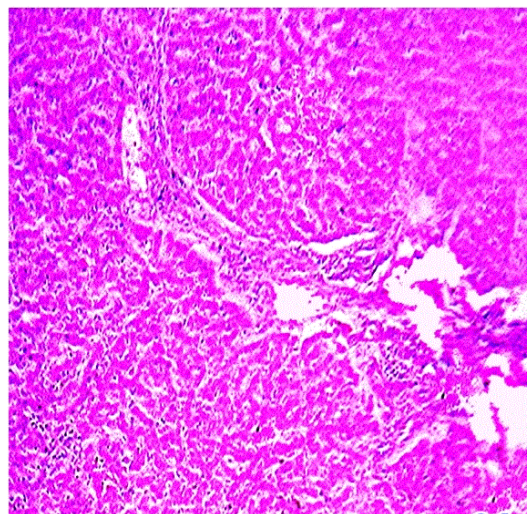


Figure 11: Lymphosarcoma: Liver: Note mild to moderate proliferation of neoplastic cells in sinusoidal spaces and necrosis of hepatic cords. H&E: 280X.

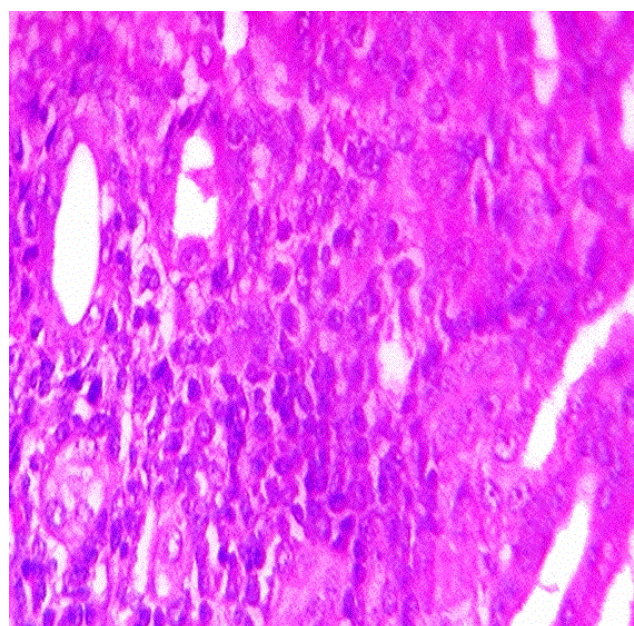


Figure 12: Lymphosarcoma: Kidney: Note mild to moderate infiltration of neoplastic cells in between the tubules. H&E: 280X.

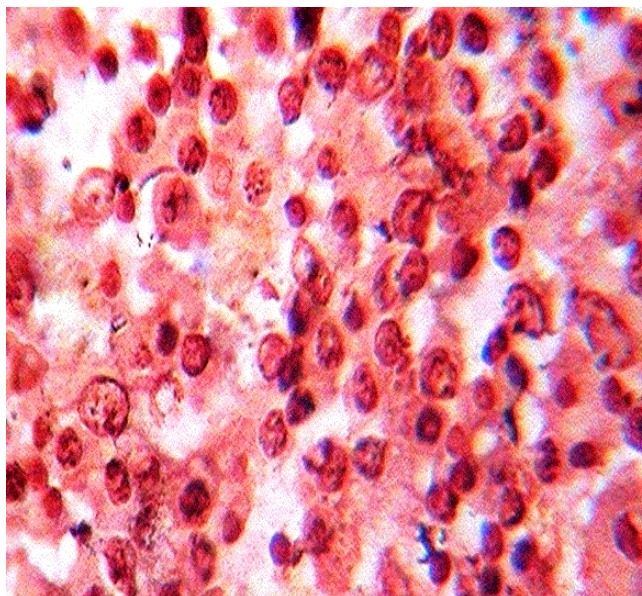


Figure 13: Lymphosarcoma: Note numerous lymphocytes and lymphoblasts with 4-6 small, irregular AgNORs throughout the nucleus. AgNOR: 700X.

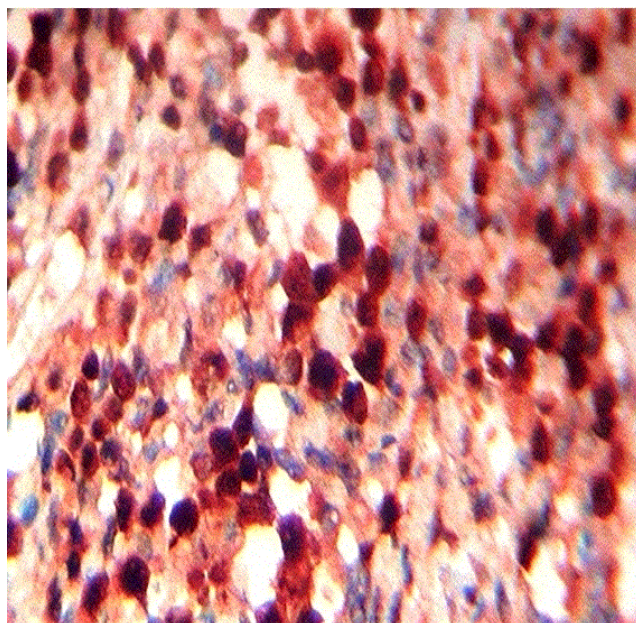


Figure 15: Lymphosarcoma: Lymphnode: IHC: Section showing uniform distribution of immunopositive neoplastic cells in the lymphnode. PCNA: 280X.

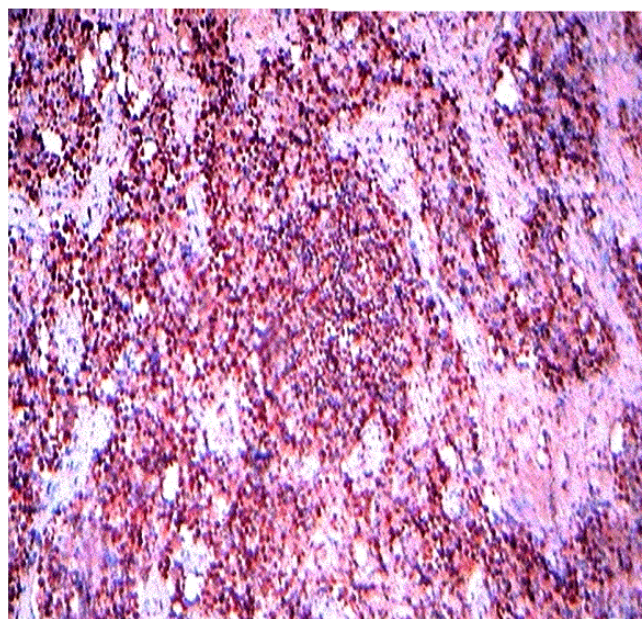


Figure 14: Lymphosarcoma: Lymphnode: IHC: Note the uniform distribution of immunopositive neoplastic cells in the lymphnode. PCNA: 70X.

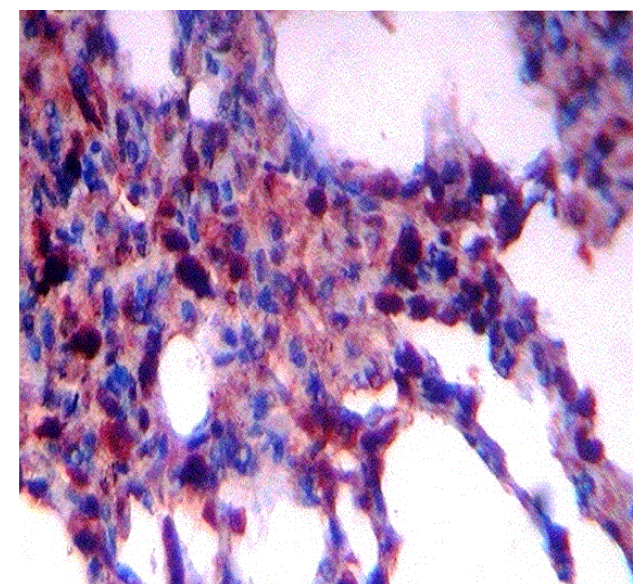


Figure 16: Lymphosarcoma: Lung: IHC: Section showing distribution of immunopositive neoplastic cells in between alveoli. PCNA: 280X.

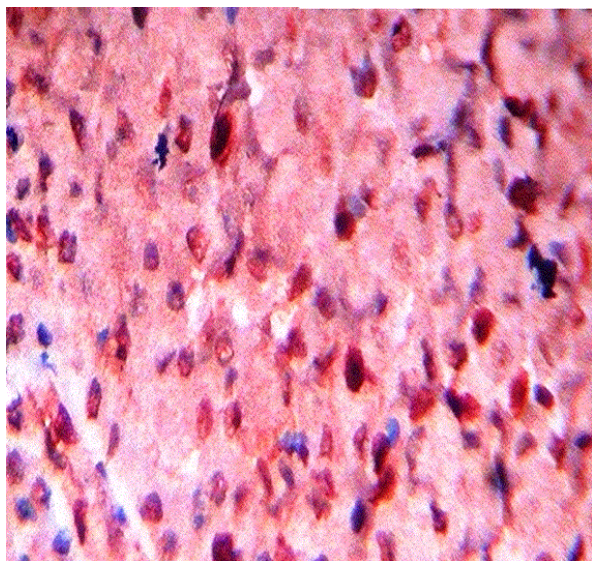


Figure 17: Lymphosarcoma: Liver: IHC: Note positive staining in few cells. PCNA: 280X.

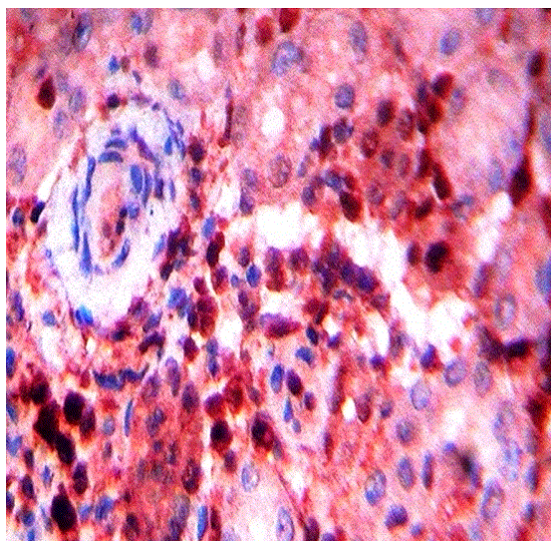


Figure 18: Lymphosarcoma: Lymphnode: IHC: Section showing uniform distribution of immunopositive neoplastic cells in the lymphnode. Ki-67: 280X.

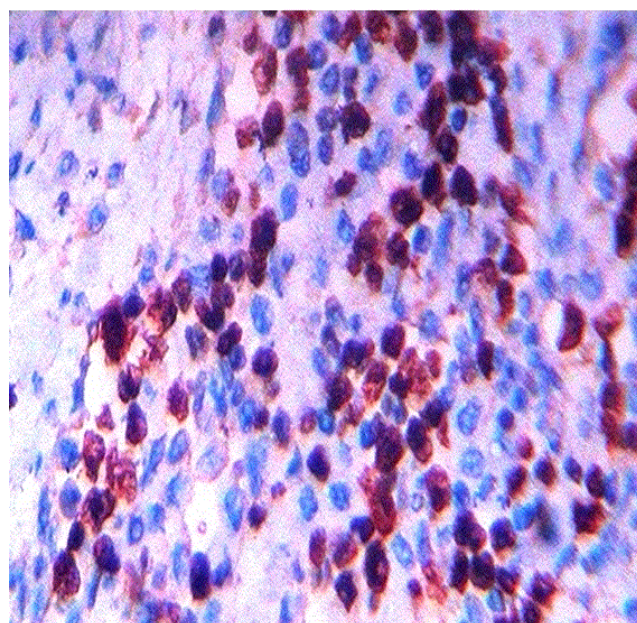


Figure 19: Lymphosarcoma: Lung: IHC: Section showing distribution of immunopositive neoplastic cells in between alveoli. Ki-67: 280X.

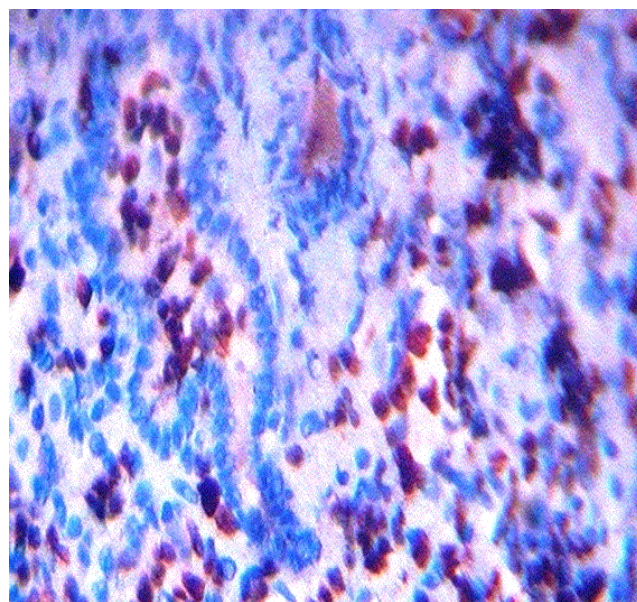


Figure 20: Lymphosarcoma: kidney: IHC: Note numerous positively stained cells in between tubular spaces. Ki-67: 280X.

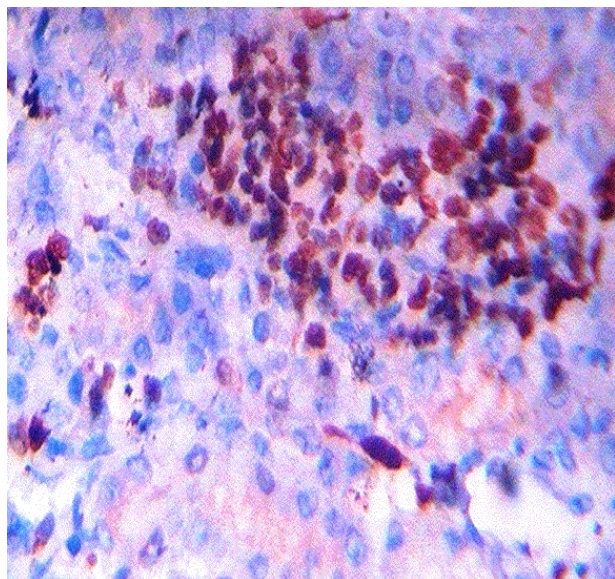


Figure 21: Lymphosarcoma: kidney: IHC: Note numerous positively stained cells in between tubular spaces. PCNA: 280X.

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