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PATHOLOGICAL AND MOLECULAR STUDIES ON AVIAN LEUKOSIS VIRUS SUBGROUP (J) IN CHICKEN PARENTS FLOCKS

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SUMMARY

The present study was carried out on 10 parent chickens flocks collected from different provinces. The examined cases were classified into 47 commercial layers and 198 broiler parents to study the gross and microscopical lesions of different tumors induced by avian leukosis subgroup-J (ALV-J) in broiler breeders and layers flocks.

Serological tests were conducted on 245 serum samples to detect the antibodies of ALV-J. Polymerase chain reaction PCR was carried out on tissue samples to confirm the presence of DNA of ALV subgroup J. Myeloid leukosis was recorded in four broiler flocks induced by the novel subgroup-J of ALV.

Pathological findings of infected parent chickens flocks revealed multiple neoplastic nodules in different organs including liver, kidney, gonads, intestine, skeletal muscle and bone. Histologically, the main constituent of tumor lesions was mature and immature myelocytes. Nephroblastoma was considered as the first recorded cases in Egyptian broiler parent flocks affected with ALV subgroup J.

The results of this work concluded that the histopathological examination plays a crucial and decisive role in diagnosing different types of neoplasm caused by avian leukosis virus subgroup-J. The application of PCR test verified the presence of DNA of ALV-J that induced the tumors of myeloid leukosis.

INTRODUCTION

Leukosis/sarcoma (L/S) group designated a variety of transmissible benign and malignant tumours of chickens caused by members that belong to the family Retroviridae Regenmortel et al. (2000). The broad host range pattern of (HPRS-103 strain) differs from those of viruses of subgroups A to G and I and provided a support for placing the strain HPRS-103 of ALV-J in a new envelope subgroup, designated as subgroup (J) Payne et al. (1992).

Enzyme linked immunosorbent assay (ELISA) was used for the detection of antibody to the gp85 envelope protein of ALV-J. The test was of value for large-scale screening programs for the presence of ALV-J infection Fuchs et al. (2000). The PCR test was considered as accurate tool for detection of subgroup- J ALV. This test depended on that the env gene sequence of ALV subgroup J was distinct from other subgroups by using a down steam primer derived from HPRS-103 env sequence. Smith et al. (1998).

Histopathological investigations revealed typical lesions of myelocytomatosis. Gross lesions of myeloid leukosis tumors were seen in several organs included liver, spleen, thymus, gonads and kidneys. The tumor masses were also observed in skeletal muscle, inner sternum, ribs, vertebrae and synsacrum Payne (1998). The morphology of the tumor cells can be verified in sections or smears stained with Romanowsky or May- Grünwald-Giemsa stains. The tumors consisted of uniformly differentiated mature myelocytes, whose cytoplasm was filled with acidophilic round granules Nakamura et al. (2000); Mona, (2000) and El-Gohary et al. (2000).

MATERIAL AND METHODS

The present study was conducted on 245 commercial layers and broiler parents received from different governorates for diagnosis of avian tumors during 2004- 2005 as shown in table (1).

Table (1) showing the number of examined birds and their geographical distribution.

Flock no.	Governorate	Type of flock	Age of bird	No. of birds
1	Giza	Broiler parent	32 weeks	23
2	Ismailia	Broiler parent	38 weeks	25
3	Behera	Broiler parent	28 weeks	20
4	Behera	Broiler parent	26 weeks	20
5	Kaliobia	Layers	36 weeks	22
6	Giza	Layers	35 weeks	25
7	Behera	Broiler parent	35 weeks	25
8	Behera	Broiler parent	30 weeks	30
9	Behera	Broiler parent	40 weeks	30
10	Dakahlia	Broiler parent	32 weeks	25

Sampling:

Blood Samples:

A total of 245 blood samples were collected from 2 commercial layers and 8 broiler parents flocks. Blood samples were left for agglutination overnight and then centrifuged at 3000 rpm /10min. to separate the serum for detection of antibodies of ALV and ALV- J by ELISA test as shown in table (2).

ELISA test for detection of ALV& ALV-J antibodies in serum samples:

A total of 245 serum samples were tested for the presence of ALV (A, B) & ALV-J antibodies. The procedure recommended by the manufacturers (IDEXX, labo-ratories, Inc., Maine, and USA) was applied according to (Venugopal et al., 1997)

Table (2): Showing the number of blood samples collected for Serological test:

Type of chickens	No. of flocks	No. of samples
Layers	2	47
Broiler parents	8	198
Total	10	245

Tissue Samples:

Tissue specimens collected from the examined chickens were divided into two parts. The first part of tissue samples were fixed in neutral buffered formalin 10% for histopathological examination. The second part of tissue samples were collected from different organs showed tumor lesions and frozen at - 70° C for polymerase chain reaction PCR test.

Histopathological examination:

Tissues from liver, kidneys, heart, skeletal muscle, ovaries, bursa of Fabricius, spleen, and intestine were taken from examined chickens and fixed in 10% neutral buffered formalin. The fixed specimens were then trimmed, washed, dehydrated in ascending grades of alcohols, cleared in xylene, embedded in paraffin, sectioned at 4-6μ thickness and stained with haematoxylin & eosin. Moreover, special stains were used. Methyl green Pyronin and May - Grünwald - Gimsa Bancroft & Cook (1993).

PCR assay for detection of ALV-J DNA genome in tissues homogenates:

PCR test was conducted on DNA extracted from tissues that showed tumor lesions in 6 flocks which given positive ELISA test for ALV-J as recorded in table (3). The concentration of DNA was determined by measuring of absorbency at 260 nm and template concentration adjusted to 50ug/ml (Murray & Thompson 1980). Reddy Mix PCR - Master Mix PCR reagent Kit with amplifier Taq DNA polymerase. (PCR reagents are manufactured by AB gene laboratories, Surrey, UK) (Lot No. 0311/10). Primers: the specific sequences of nucleotides for ALV-J used in the study forward & reverse were H5/H7 respectively as described by (Smith et al., 1998).

PCR program was consisting of the following steps: The amplification: denaturation at 93°C for 1min., annealing at 60°C for 1min. decreasing by 1°C in each cycle and extension at 72°C for 90 seconds to 13 cycles followed by 30 cycles of 93°C for 1 min., 48°C for 1 min., 72°C for 90 sec. with final extension at 72°C for 10 min. Reactions were conducted in Thermocycler. The amplification products were analyzed by gel electrophoresis was prepared by 1.5 gm ultra pure agarose added to 100ml TBE then melted in hot air oven. Products were stained with ethidium bromide (0.5 ul per ml of gel). The stained amplified products were observed under ultra violet transluminator and photographed by Polaroid Camera (Smith et al. 1998).

Table (3): Showing the number of examined flocks for DNA detection of ALV-J using PCR test:

Type of chickens	No. of examined flocks
Layers	2*
Broiler parents	4**
Total	6

* Flocks no. 1, 2, 4 and 10.
** Flocks no. 6,7.

RESULTS

Pathological findings:-

Liver:

The liver of infected birds was greatly enlarged occupying most of the abdominal cavity. Multiple elevated creamy nodular subcapsular masses were seen (Fig. 1).

The hepatic lobules showed focal aggregations of mature and immature granulated myelocytes adjacent to the blood vessels (Fig. 2). The hepatocytes were replaced by neoplastic cells with atrophy of hepatic cords. The portal triads were infiltrated with large number of proliferating myelocytes. The bile ducts were lined by cuboidal or columnar epithelium and mucous secreting cells (Fig. 3). These myelocytic cells were characterized by eccentrically located nuclei and marked nucleolus with clear mitotic figure. The cytoplasm was filled with conspicuous spherical eosinophilic granules.

Kidneys:

The kidneys of infected birds showed grayish white patches of variable size and shape. Subcapsular haemorrhagic areas were also seen. The kidney appeared friable with marked enlargement of its lobes compared with non infected birds (Fig. 4).

Microscopically, kidneys of infected birds showed focal aggregations of immature and mature granular myelocytes in both cortex and medulla. The tumor cells were aggregated usually around the dilated blood vessels (Fig. 5). The renal tubules showed sloughing of its epithelial lining together with the presence of flocculated

intra-luminal protineous material. Some renal tubules showed cellular cast consisted of desquamated epithelial cells intermingled with myelocytes (Fig.6).

Heart:

The thoracic cavity showed yellowish white, soft and friable nodular or diffuse masses. These tumour masses were seen in costochondral junctions of the ribs, the inner surface of sternum and cardiac muscle (Fig.7).

The cardiac muscle showed nodular lesions consisted of massive aggregations of immature and mature granular myelocytes. Perivascular oedema with myeloid cells infiltration was also seen (Fig.8).

Skeletal Muscles:

The skeletal muscles adhered to the flat bones especially the sternum, ribs and synsacrum revealed grayish white, soft and friable elevated nodules of variable sizes (Fig.9).

Stained section of the pectoral muscle of the infected birds revealed Zenker's necrosis of muscle bundles which appeared as homogenous eosinophilic structureless masses separated by mature and immature myelocytic cells infiltrations (Fig.10).

Bone and Cartilage:

Some infected birds showed typical features of myelocytomatosis characterized by yellowish white nodules on the surface of sternum, ribs and synsacrum.

The cartilaginous part of sternum revealed large numbers of proliferating mature and immature granulated myelocytes in diffuse manner around the degenerated cartilage (Fig.11).

Intestine:

The intestinal mucosa revealed grayish white elevated areas scattered along the intestinal tract. The intestinal lumen was filled with yellowish slimy fluid with offensive odour.

Microscopically, the intestinal mucosa showed sloughing of its epithelial lining. The lamina propria was infiltrated with large number of mature and immature myelocytes. The central lactael were dilated and surrounded by uniformly differentiated mature myelocytes (Fig.12). The submucosal connective tissue showed perivascular oedema and also myelocytic infiltration.

Ovary:

Most of the examined cases were suffering from decreased egg production associated with atrophy of ovarian follicles (Fig.13).

The ovary of infected bird showed focal aggregations of mature and immature myelocytic cells which occupied most of the ovarian parenchyma. The myelocytic cells surround the atrophied ovarian follicles and perivascular spaces (Fig.14).

These myelocytic cells showed a characteristic brilliant red colored cytoplasmic granules when stained with May - Grünwald - Gimsa stain) (Fig. 15).

Miscellaneous tumor

Nephroblastoma:

Nephroblastoma was considered as highly malignant embryonic tumor. This type of tumor was recorded in two examined birds infected with ALV-J. The kidneys of infected birds showed spherical well demarcated tumor mass embedded in its parenchyma. On cut section, the tumour appeared soft, friable, grayish white with hemorrhagic spots.

The tumour mass was separated from apparently healthy tissue by thick fibrous connective tissue and leukocytic infiltration (Fig.16). The neoplastic mass showed undifferentiated cystic renal tubules with intra-luminal protineous cast (Fig. 17). The interstitial tissue showed polymorphic stromal cells which appeared round or stellate in shape. Structures like glomeruli or metanephric precursor of glomeruli were observed. The renal tubules were lined by undifferentiated cuboidal or columnar epithelium and merged with stromal cells (Fig. 18).

Results of serological test (ELISA):

ELISA test was applied on 245 serum samples 47 of commercial layers and 198 of broiler parents for detection of ALV-J antibodies. The age was ranged from 26 up to 50 weeks. ELISA test revealed positive results in 6 out of 8 broiler parent flocks with an incidence of (19/23) 83%, (20/25) 80 %, (14/20) 70%, (15/20) 75%, (21/30) 70% and (18/25) 72% respectively and a total incidence (107/245) 45.7% among broiler chicken. On the other hand commercial layers reacted negatively with (ELISA) test as shown in table (4).

Table (4): Results of examined sera samples for detection ALV- J antibodies by using ELISA:

No.	Province	Type	Age (weeks)	No. of samples	Results		
					No. of positive	* S/P mean for positive	%
1	Giza	BP	32	23	19	1.023	83
2	Ismailia	BP	38	25	20	0.980	80
3	Behera	BP	28	20	14	0.850	70
4	Behera	BP	26	20	15	0.905	75
5	Kaliobia	L	36	22	0	-	0
6	Giza	L	35	25	0	-	0
7	Behera	BP	35	25	0	-	0
8	Behera	BP	30	30	0	-	0
9	Behera	BP	40	30	21	0.845	70
10	Dakahlia	BP	32	25	18	0.966	72
Total				245	107		43.7

*S/P ratio greater than 0.6 indicated presence of ALV-J antibodies.

Results of ALV-J DNA detection in tissues using PCR test:

PCR test conducted on DNA extracted from tissues showed tumor lesions from the tested flocks. PCR test revealed positive amplification of 545 bp fragment with the extracted DNA of ALV-J in lanes (4, 5, 6, and 7) and negative amplification in lanes (8 and 9) for 2 flocks as shown in photo (1). Four broiler parent flocks were positive PCR test out of six flocks positive for ELISA test as shown in table (5).

Table (5): Results of PCR test for detection DNA of ALV-J in tissues:

No.	Governorate	Age	Type	PCR results
1	Giza	32	BP	+
2	Ismailia	38	BP	+
3	Behera	40	BP	+
4	Dakahlia	32	BP	+
5	Behera	35	BP	-
6	Giza	35	L	-

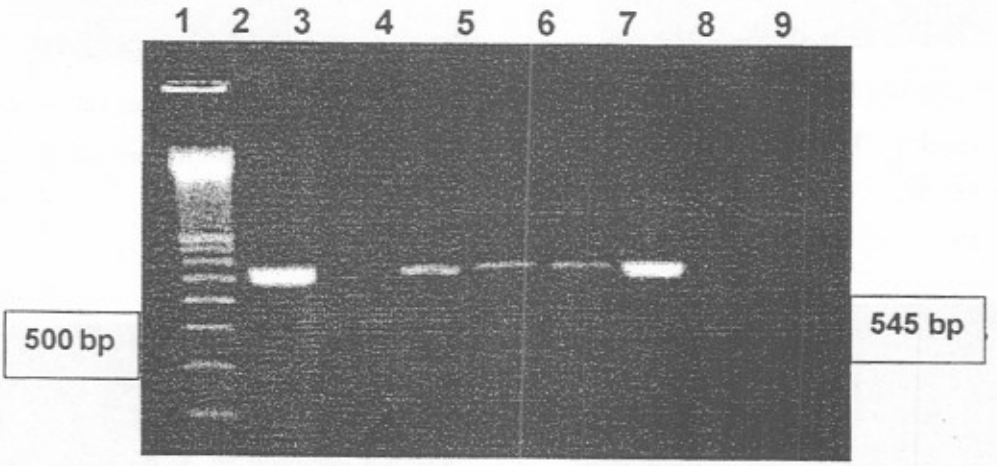

Photo (1): Electrophoresis of PCR on 1.5% agarose gel stained with ethidium bromide showing:

* 100 bp molecular weight ladder (lane 1).
* Positive control amplified DNA products at 545 bp (lane 2).
* Negative control (lane 3).
* Amplification products at 545 bp fragment indicating DNA of ALV - J with H5, H7 primers (lanes 4, 5, 6 and 7).
* Samples (lanes 8, 9) indicating negative amplification.

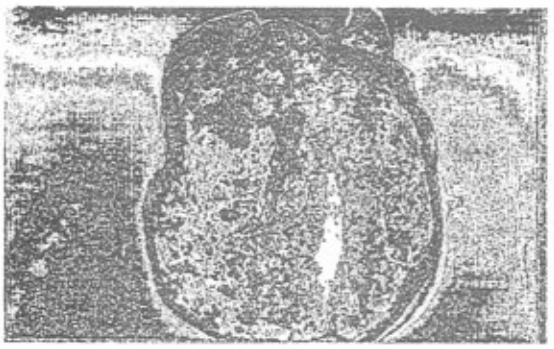

Fig. (1): Liver showing diffuse enlargement with white creamy nodules on its surface.

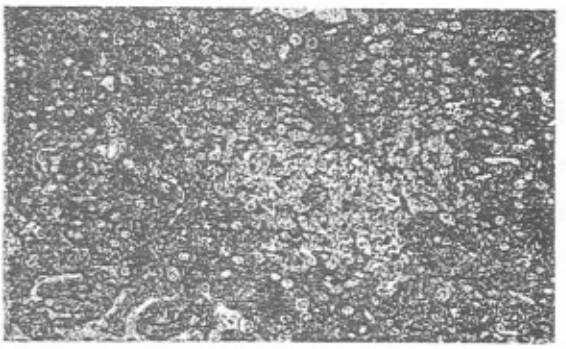

Fig.(2): Liver showing perivascular myelocytic aggregation (H & E x400).

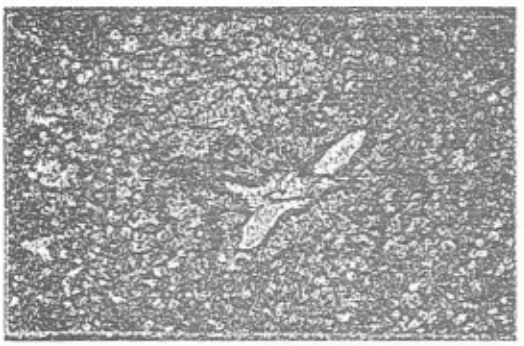

Fig. (3): Liver showing hyperplasia of bile duct surrounded by myelocytic cells (H&Ex400).

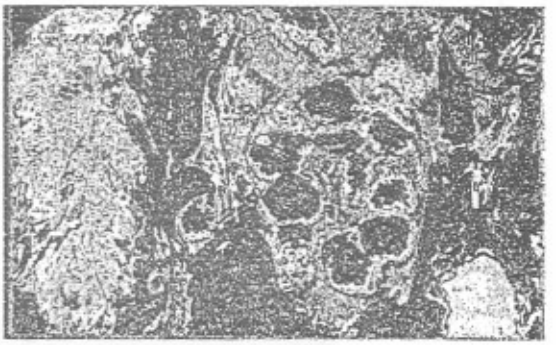

Fig. (4): Kidney showing diffuse enlargement with greyish white patches and subcapsular hemorrhages.

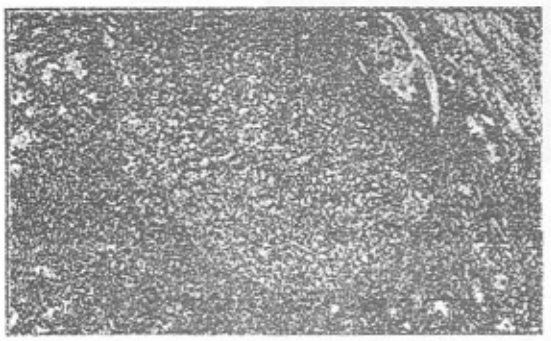

Fig. (5): Kidney showing focal aggregation of myelocytes surrounding dilated blood vessels (H&Ex100).

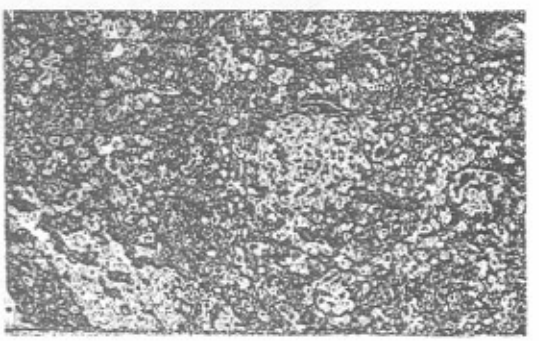

Fig. (6): Kidney showing intra-tubular cellular cast composed mainly of myelocytes and epithelial cells (H&E x 200).

Fig. (7): Thoracic cavity showing white to creamy color nodules on the inner surface of sternum and heart.

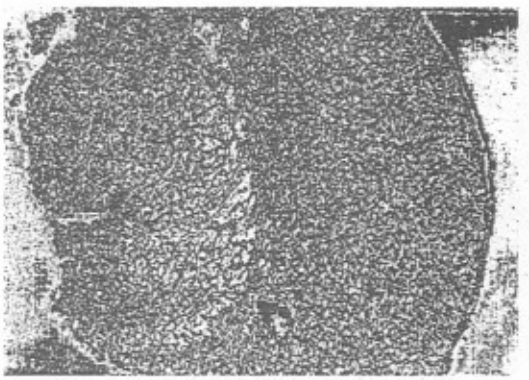

Fig. (8): Heart showing diffuse aggregation of myelocytic cells (H&E X 40).

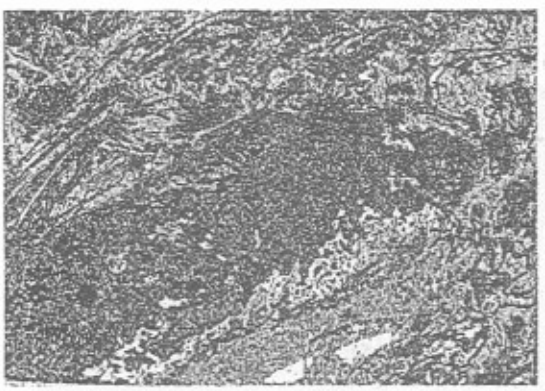

Fig. (9): Skeletal muscle showing multiple greyish white nodules

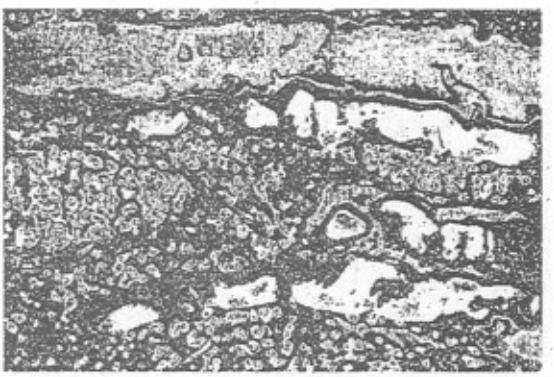

Fig. (10): Skeletal muscle showing Zenker's necrosis and myelocytes infiltration (H&E X 400).

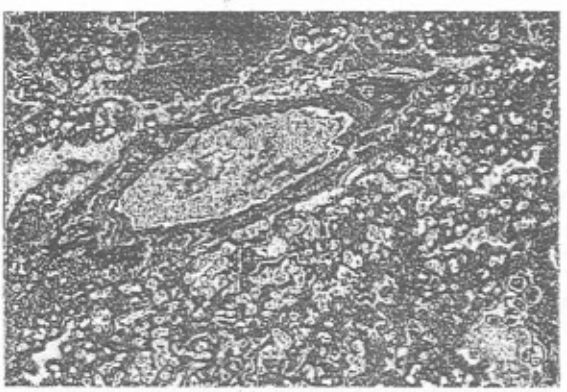

Fig. (11): Sternum section showing diffuse infiltration of myelocytes around degenerated cartilage (H&E X 400).

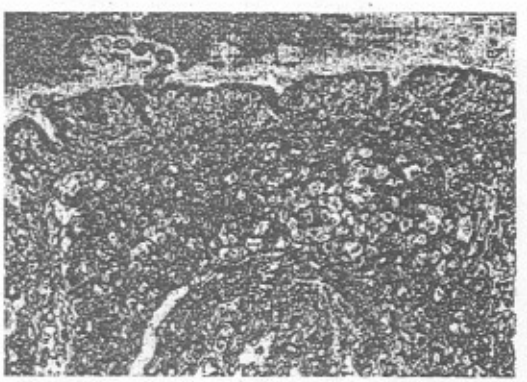

Fig. (12): Intestinal mucosa showing massive infiltration of myelocytes (H&E X 200).

Fig. (13): Abdominal cavity showing diffuse enlargement of kidney lobules and ovarian atrophy.

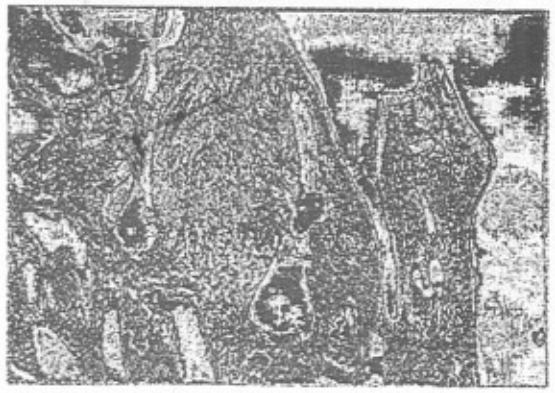

Fig. (14): Ovary showing atrophy of its ovarian follicles with focal aggregation of myelocytes (H&E X 100).

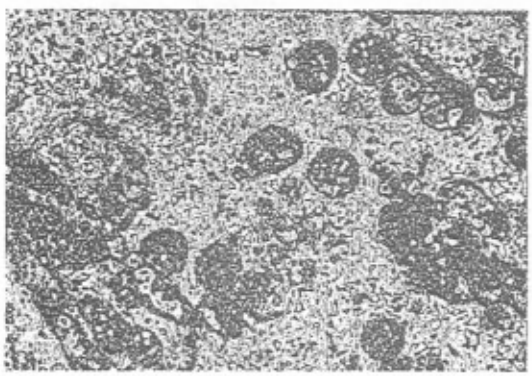

Fig. (15): Ovary showing myelocytes with brilliant red cytoplasmic granules stained with May Grun-wald Gimsa (X 400).

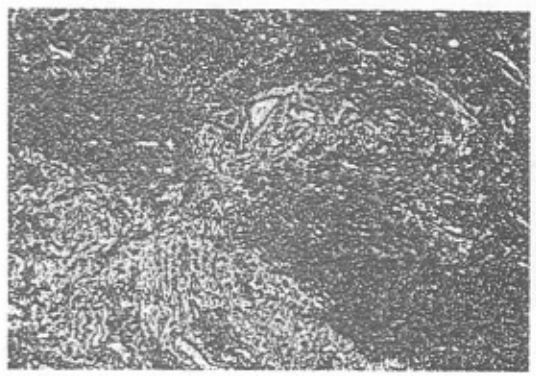

Fig. (16): Nephroblastoma showing herniation of tumor through thick fibrous connective tissue to healthy part (H&E X 200).

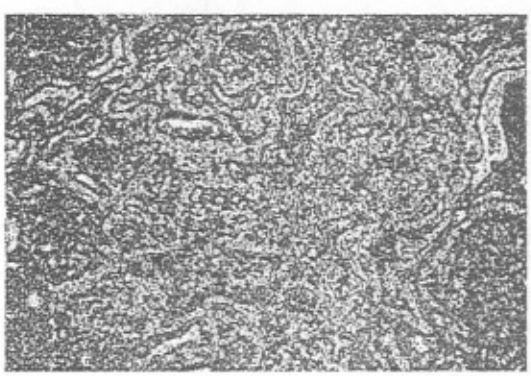

Fig. (17): Nephroblastoma showing undifferentiated basophilic tubular epithelial lining (H& EX 200).

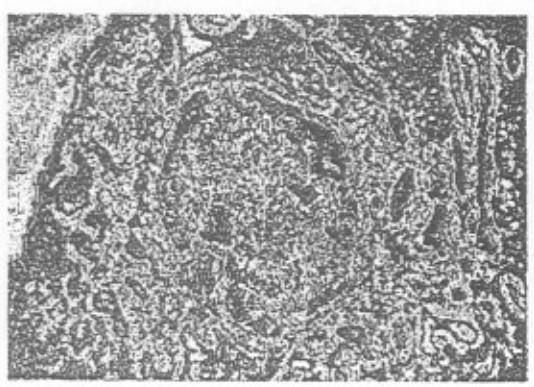

Fig. (18): Nephroblastoma showing primitive glomerulus (H&E X 200).

DISCUSSION

The present work was designated for diagnosis of avian leukosis virus subgroup J in both layers and broiler parents. The investigations of infected chicken flocks based on pathological, serological, and molecular biological methods.

Myeloid leukosis was associated with the infection of a novel subgroup of ALV designated J in meat type chickens in United Kingdom as firstly recorded by Payne et al. (1991). On the other hand ALV-J was recorded in broiler breeder flocks in Egypt by Mona, (2000).

Myeloid leukosis virus spreads vertically and horizontally leading to severe damage in the poultry industry. Recently, the sequence changes of env gene in variable regions caused rapid variation of the antigenicity of ALV-J leading to emerging of variant viruses due to the antigenic variation as elucidated by Venugopal et al. (1998).

The transforming strains of ALV that induce myelocytomatosis, such as MC29 and CMII, which carry the v-myc oncogene was reported by Enrietto and Hayman (1987). Slowly transforming strains of ALV subgroup- J that also induce myelocytomatosis, such as HPRS-103 and ADOL-Hc1, do not carry an oncogene, but molecular studies of HPRS-103 that induced myelocytomatosis indicated that c-myc was activated (Chesters et al.,2001). The acutely transforming strain 966 ALV, derived from myelocytoma and induced by strain HPRS-103 of subgroup- J ALV, has been shown to carry v-myc as reported by Payne et al. (1993).

Arshad et al. (1997) concluded that HPRS-103 showed a lower propensity to replicate in the medullary region of the lymphoid follicles of fabricius bursa more than RAV-1 strain of subgroup-A avian leukosis virus. This low bursal tropism may be a factor in why HPRS-103 did not induce lymphoid leukosis. This strain of subgroup-J replicated in blood monocytes cultures from chickens indicating a tropism for the myelomonocytic cell linage.

Studies on HPRS-103 and 966 showed that they have a tropism for the myelomonocytic cell lineage rather than the lymphoid cell lineage, which may related to their ability to cause myelocytomas as reported by Arshad et al., (1999).

Diagnosis of avian leukosis virus subgroup-J in commercial brown egg layers in China was based on observations of gross lesions, histopathology, and PCR tests. The affected birds showed yellowish/ white tumors which were observed on the visceral surface of the sternum in nodular form Binrui Xu et al. (2004).

Our serological diagnosis of suspected birds by using ELISA test revealed that 6 flocks were

positive for ALV-J antibodies by different percentage and titer. Presence of ALV-J antibodies indicated that, these flocks exposed either to the current or past infection. The rest 4 flocks were negative for ALv-J antibodies. This serological method was recommended by Venugopal (1999).

Detection of ALV-J DNA in tissues (liver, kidney, and ovary) of infected birds was applied by using PCR test. The results of polymerase chain reaction gave the confirmation to the results of histopathology for myeloid leukosis. It gave positive amplifications at 545 bp to the 4 flocks. These results coincide and came parallel with the data reported by Smith et al. (1998), and Binrui et al. (2004).

The first alterations of ALV subgroup- J occurred in bone marrow, which characterized by overcrowding of intra-sinusoidal spaces with two types of cells, the myeloid stem cell and the neoplastic myelocytes. The latter appears to arise directly from the stem cell and differentiation is arrested both non-granulated and granulated myelocytes. Tumors formed by expansion of marrow growth and may crowd through the bone and periosteum. Extramedullary tumors may also arise by blood-borne metastasis as stated by Payne and Fadly (1997).

Our results revealed that myeloid tumours, grossly appeared as dull, grayish white or creamy white, friable to cheesy and nodular or diffuse in manner. These findings come in agreement with Payne et al. (1991).

Histopathologically, myeloid tumours consisted of uniformly arranged myelocytes with very little stroma so, the myeloid tumours are characterized by high cellular and less stromal connective tissue.

The myelocytic cells characterized by large vesicular eccentrically located nucleus. The cytoplasm was tightly packed with eosinophilic granules which were usually spherical. When these cells stained by May- Grünwald- Gimsa the granules appeared brilliant red or purple. These findings come in agreement with that reported by Mladenov et al. (1967).

Livers of infected birds were slightly swollen with mottled grayish/white pinpoint spots under the capsule. Spleens were enlarged either slightly or may reach to several times of normal and had yellowish/ white tumor nodules in a few cases. Kidneys were markedly swollen, and some had light grayish/ white mottled tumor masses. Ovaries and oviducts of hens were undeveloped at 170 days of age. These findings were agreed with Sultan et al. (2004).

Myelocytic cells were aggregated near blood vessels and portal triads then invaded the hepatic cords. Also; myelocytes were replacing the hepatocytes leads to degenerative changes and atrophy of the hepatic cells. The principal

phenomena of this pattern are the formation of invasive growth in the parenchymatous organs. These findings are similar to that reported by Beard, (1980).

Tumor cells were present around veins and arteries in the liver and grew focally. In the spleen, lymphocytes decreased and tumor cells were widely present in both the red pulp and the white pulp. Epithelial cells of renal tubules were swollen, degenerated and separated from the basement membrane. Proliferated tumor cells grew focally in the ovary and oviduct. Few myeloid tumors were observed in the myocardium. These findings coincide with that observed by Binrui et al. (2004).

Myeloid tumors were seen in periosteum and near the cartilages, therefore any adjacent tissue or organ may be affected. Tumors often developed at the costochondral junction of the ribs and inner sternum. Flat bones of the pelvis and synsacrum were also affected. These findings coincided with Payne et al. (1991) and Payne & Fadly (1997).

Avian lecukosis virus subgroup-J was recorded in white Leghorn egg layer flocks being used to produce fertile eggs for human vaccine production exhibited dramatically in low egg production and high number of non- laying birds after the onset of sexual maturity. Gross lesions of freshly dead birds necropsied revealed lacking ovarian activity approximately 60% and had lesions of bacterial bursitis or synovitis, whereas the other 40% had tumors of the viscera but not of the bursa of fabricius Gingerich et al. (2002). They also suggested that hatching of day-old egg type chicks with ALV-J infected meat-type chicks in a common hatchery had contributed to cross infection.

Nephroblastoma is considered as embryonal highly malignant tumour. This type of tumour was recorded in two examined birds infected with ALV-J. The kidneys of infected birds showed spherical, well demarcated tumor mass that replaced part of the parenchyma. On cut section, the tumor appeared soft, friable, grayish white with haemorrhagic spots.

Microscopically, the tumor mass was separated from apparently healthy tissue by thick fibrous connective tissue and leukocytic infiltration. Structures like glomeruli or metanephric precursor of glomeruli were observed. The interstitial tissue showed polymorphic stromal cells which appeared round or stellate in shape. The renal tubules were lined by cuboidal or columnar epithelium with hyperchromatic nuclei. The epithelium of the renal tubule was merged with stromal cells. This finding comes in accordance with Payne et al. (1993).

This study concluded that the histopathological examination plays a crucial and decisive role in diagnosis among different types of neoplasm

caused by avian leukosis virus due to different target tissue affections. Application of specific stains for granular myelocytes was aid for differentiation between the lymphoid and myeloid leukosis.

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