

EVIDENCE FOR VARIANT AVIAN LEUCOSIS VIRUS IN CASES OF MIXED INFECTION WITH MAREK'S DISEASE VIRUS

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SUMMARY

In the present study, we report for the first time in Egypt the evidence for variants of avian leucosis virus subgroup J. ALV-J associated with 2 cases of mixed infection with Marek's disease virus, MDV (one case is associated with tumors and the other from homogenates of blood and tissues of chicks experienced transient paralysis syndrome of MDV) was detected. The ALV-Js might be variants as indicated by histopathological behaviour; negativity of the ALV-J specific PCR; and serological profile in the flock with mixed infection (tumor-cases of ALV and MDV) and in experimentally-infected chickens with blood and tissue homogenates of other mixed infection case of ALV-J and MDV was detected. Using specific PCR for REV and ALV groups A or B, or C and/or D, all samples of both mixed infection cases

revealed negative results for amplification. However, 28.3% of sera samples collected from chickens with tumor-cases at age of 38 weeks were positive for ALV-J and negative for ALV (groups A and B) by ELISA. Testing of sera collected from the flock with tumor-cases at 45 and 55 weeks of age revealed that 38.2% and 20% of sera were positive for ALV-J and 2.9 and 3% were positive for ALV (groups A and B), respectively. In experimentally-infected chickens with homogenates of blood and spleen, 14.2 % of sera were positive to ALV-J and negative for ALV (groups A and B) when tested at 30 weeks post-inoculation. Histopathological examination revealed the occurrence of mixed infection of MDV and ALV-J with unique pathological lesions in eye and liver which were found to have heavily aggregations of myelocytes characteristic to ALV subgroup J.

INTRODUCTION

In the 1990s, after eradication programmes of exogenous avian leucosis virus (ALV) have been carried out by commercial poultry breeding companies, ALV subgroup J (ALV-J) emerged and caused myeloid leucosis (ML) in meat type chickens with economic losses worldwide in broiler breeder stocks (Payne, 1998, Fadly and Smith, 1999 and Fadly, 2000). ALV-J commonly induces myeloid leucosis (myelocytomatosis) in naturally and experimentally infected flocks (Payne et al., 1992). This is due to its ability to replicate well in blood lymphocyte than in the bursal follicle (Arshad et al., 1997). HPRS-103 strain of ALV, isolated in 1988 from meat-type chickens, is the prototype of the ALV-J (Payne et al., 1991).

HPRS-103 genome has a distinct env gene closely related (75%-97% similarity) to a novel group of endogenous retroviruses designated EAV-HP than to env gene of other ALV subgroups (40% similarity) (Bai et al., 1995; Smith et al., 1999). Such similarity support the hypothesis that ALV-J arose by recombination between unidentified exogenous ALV and an EAV (Lupiani et al., 2003). EAV-HP (Benson et al., 1998), ev/J (Smith et al., 1999) and line 0 env-like (Silva et al., 2000) are the three classes of the newly identified family of endogenous viruses. Significant antigenic variations have been observed among many isolates of ALV-J (Fadly and Smith, 1999, Hunt et al., 1999, Payne, 1998) which is probably because these iso-

lates inherently have an unusually high frequency of mutation (Silva et al., 2000, Venugopal, 1999, Venugopal et al., 1998). Recently, many recombinants ALV-J viruses carrying a subgroup A envelope glycoprotein (gp85) have been isolated and molecularly characterized (Hunt et al., 2000, Lupiani et al., 2000). These recombinants were accidentally generated between two ALV-J isolates and a defective subgroup A ALV present in transgenic chicken line alv6 (Crittenden and Salter, 1992, Fedrspiel et al., 1991). It is well documented that ALV-J viruses may easily recombine with endogenous viruses and further genetic shift and drift may occur generating new viruses with new phenotypes such as host range, antigenicity and pathogenicity (Lupiani et al., 2000). The characterization of these variants are very important in the diagnosis of ALV-J infection by neutralization or DNA detection by PCR as they may have negative effect on the efforts to control the infections in the primary breeding stocks (Venugopal et al., 1998, Fadly and Smith, 1999, Silva et al., 2000 and Lupiani et al., 2000).

The aim of the present study is to detect and characterize avian leucosis virus associated with 2 cases of MDV infections (tumor and transient paralysis syndrome) by PCR, histopathology and serology.

MATERIAL AND METHODS

Case History: First case: In 2004, chicks with transient paralysis syndrome (difficulty in move-

ments, neck and leg paralysis with mortalities which lasted for 2 weeks have been submitted for the laboratory for diagnosis of the possible causative pathogen. Using PCR and histopathology, pathogenic MDV was implicated as the cause of the problem. MDV was isolated and neurotyped as very virulent plus strain of MDV (Hussein et al., 2004). Examination of different organs from SPF chicks experimentally infected with the blood and brain and feather follicle homogenates collected from the original infected chicks by PCR and histopathology confirmed MDV infection. In a trial to evaluate the protection induced by the current MDV vaccination strategy against the isolated MDV, different groups of commercial broiler breeder chicks have been used in 2 months experiment. PCR and Histopathology examination of organs collected from those broiler breeder chicks at the end of experiment indicated lesions of MDV. Universal PCR to amplify all groups of ALVs including endogenous viruses revealed positive results in spleen collected from inoculated chicks. Therefore we kept the inoculated groups till 30 weeks of age to investigate the possibility of the occurrence of mixed infection of MDV and ALV in the original chicks. Three groups of broiler breeder chicks were kept under investigation: Group 1 and 2 were MDV vaccinated at hatch and non vaccinated chicks kept for 10 days before get challenged by blood, brain and feather follicle homogenates of the original flock via intra-

peritoneal route. Group 3, were kept non vaccinated and non challenged chicks as control. The chicks were kept for 208 days (almost 30 weeks). Blood, sera and different visceral organs were collected at 6 and 30 weeks and subjected for histopathology, serology and PCR. Second case: In 2005, tumor cases from 30-weeks-old, female broiler breeder flock were submitted for laboratory diagnosis. The tumors were mainly visceral form (liver and spleen). Visceral organs, brain, eye and sciatic nerves were collected from the necropsied chickens as well as blood samples with and without anticoagulants were collected from the original flock for PCR, serology and histopathology examination. Sera were also collected from the same flock at 38, 45 and 55 weeks of age for serology testing by ELISA (ALV, groups A and B and ALV-J antibodies against gp 85).

Histopathology examination: Different organs were fixed in 10% buffered neutral formalin solution, processed in normal way, paraffin sections (4-6 micron thickness) were prepared and stained with haematoxylin and eosin (Bancroft, 1996).

Extraction of viral DNA and PCR : Eight PCR assays with primers specific for MDV, REV, ALV (universal for all subgroups A to E), ALV-A, ALV-B, ALV-C, ALV-D and ALV-J were applied to detect the corresponding viruses in the collected samples (blood and spleen).

The primers used and PCR conditions were carried out as previously reported for MDV serotype 1 using MDVF and MDVR primers which could differentiate between pathogenic (yield 302bp and/or 434bp and/or 566bp) and vaccine (yield 302bp + multiple 132bp repeats) strains of MDV (Davidson and Borenshtian, 2002), REV using primers to amplify products with size 291 bp (Davidson et al., 1995), ALV (subgroups A to E) using H5 and AD-1 primers yielding 360bp products, ALV-A using H5 and CAP-A primers to amplify products with size 694 bp, ALV-B using H5 and envB primers to amplify products with size 846 bp, ALV-C using H5 and envC primers to amplify products with size 859 bp, ALV-D using H5 and envD primers to amplify products with size 797 bp, ALV-J using H5 and H7 primers to amplify products with size 545 bp (Sequences of the primers used for ALV viruses were kindly supplied by Dr. Kirk Patrick, Ross breeders, Avigen Ltd, Scotland, UK). DNA was extracted from the samples using QIAamp DNA and DNA blood mini kits (Qiagen GmbH, Germany). The extraction protocols were carried out as recommended by manufactures. The PCR mixture reactions were ready to use master mix (ABgene, UK). The cycling conditions of each PCR assay was employed according to the type of primers used in the assay. The PCR products were analysed by electrophoresis on agarose gels stained with ethidium bromide and photographed using Polaroid camera.

Serology: ALV and ALV-J antibody test kits (Idexx Laboratories, Inc, USA) were used for the presence of antibodies against group A and B (ALV-antibody test kit subgroup J (ALV antibody test kit-subgroup chicken sera. ELISA was carried out as recommended by manufactures.

RESULTS

Confirmation of mixed infection of MDV ALV in the two cases in the current study based on the obtained results of histopathic and PCR (Figure 1, Plates 1, 2 and 3). Examination of the organs and blood of the experimentally infected commercial broiler breeder chicks (ure 1) after 6 weeks post inoculation with the progenates of the first case (chicks showing silent paralysis syndrome) by PCR revealed positive results for pathogenic MDV serotype as shown in figure 1 E. Testing those birds weeks post inoculation for ALVs by PCR as specific to each subgroup (A, B, C, D and J) revealed negative results. Testing the same extracted DNA by PCR using universal primers that amplify fragments from all subgroup (A to J) revealed positive results. Serological testing the same birds by ELISA at 30 weeks revealed positive results for ALV-J in a percentage of 2/14 and negative for ALV subgroup A antibodies.

Histopathological examination of the organs collected from the experimentally infected chicks revealed the occurrence of mixed MDV and ALV-J lesions with marked score lesions of the aggregated tumor cells in eye, liver and proventriculus

compared with the absence of the tumor lesions in the third group (non vaccinated non infected). The observed eye and liver lesions suggested that the case is a mixed infection of MDV and ALV-J based on the presence of focal and diffuse aggregates of pleomorphic lymphocytes as well as myelocytes in the oedematous iris (Plates 1 and 2). The retina showed massive congestion with haemolysis of the choroids in addition of degeneration of the photo receptor (Plate 1). The ciliary processes in group 3 showed heavily aggregation of myelocytes with obvious mitotic division of the nuclei (Plate 2). Mean while pleomorphic lymphocytes were aggregated in the conjunctiva of gpl (Plate 2). The liver lesions were in the form of focal parenchymal infiltration of small lymphocytes with few lymphoblasts. Perivascular aggregation of pleomorphic lymphocytes and great population of myelocytes were observed (Plate 2). Dialation of the hepatic blood vessels and sinusoids with thrombus formation as well as hepatocellular necrosis (Plate 1). The Proventriculus exhibited hyperplasia of the mucosal epithelium and submucosal oedema (Plate 1). Submucosal and glandular infiltration of lymphocytes, lymphoblasts and reticuloendothelial cells were observed. The severity of the scors lesions of the

aggregated tumor cells was high (++++) in MDV-non vaccinated than in MDV-vaccinated groups (+++) challenged with the homogenates specially the eye, liver and proventriculus.

PCR and histopathology examination of the tumor cases from the second case in the study revealed also mixed infection of MDV and ALV. PCR confirmed the presence of pathogenic MDV and as well as ALV (subgroups A to E) (Plate 3 G). Employing PCR assays specific for each subgroup (A, B, C, D) indicated that the samples were negative. However, the samples revealed positive amplification when universal PCR assay for all subgroups of ALV including E was applied (Plate 3H). Testing of serum samples collected from the flock at different ages post onset of tumor cases revealed positive results for ALV-J in a percentage of 28.3%, 38.2% and 20% at 38, 45 and 55 weeks of age, respectively. On the other side, testing of those sera for ALV (groups A and B) revealed low levels of antibodies 0%, 2.9% and 3% at 38, 45 and 55 weeks of age, respectively. Histopathology examination of the tumors suggested both ALV and MDV. Plate 3 represent the histopathology changes suggested that ALV is implicated. There was diffuse and massive number of proliferated and infiltrated lymphoid as well as myeloid cells which replaced most of the hepatic tissue with atrophy of the remained hepatocytes associated with sever dilatation of the hepatic sinusoids which were impacted

by massive number of erythroid cells as well as myeloid cells.(Plate3D). Also, kidney lesions showed dialation of the intertubular blood vessels and capillaries and impacted by erythroid cells in association with infiltration of the myelocytes and lymphocytes in the stromal tissue. Infiltrated myelocytes and lymphoid cells were also observed in intestine and ovary. Massive numbers of erythroid cells were seen in brain, spleen and ovaries.

On the other side, MDV lesions were seen in the same tumor tissues specially in liver and spleen where perivascular in central and portal veins and sinusoids in liver as well as lymphoid cells proliferation mainly pleomorphic lymphocytes and plasma cells in liver and spleen were observed. The histopathology examination of the tumor tissue suggested the mixed infection ALV and MDV.

Figure (1)

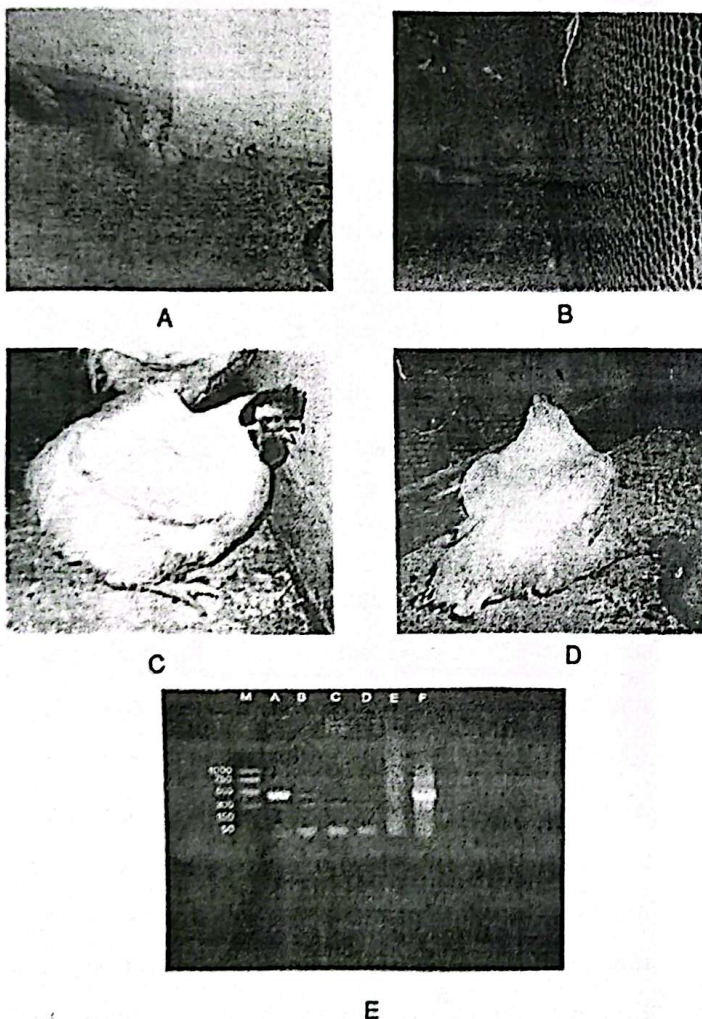
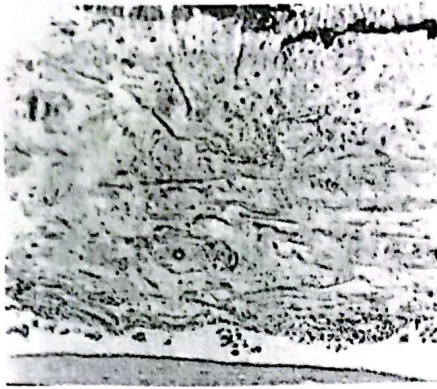
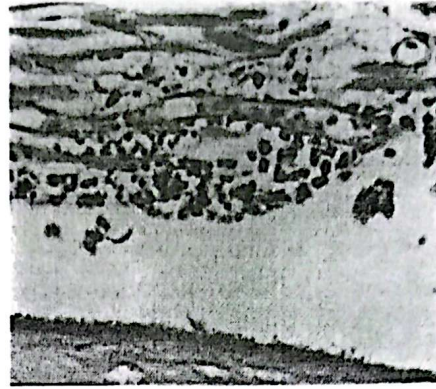


Figure 1: A and B are the groups of broiler breeder chicks used in the experimental infection with the homogenates of commercial broiler breeder chicks with acute transient paralysis syndrome. C and D, the inoculated chicks after 208 days showing some of leg problems and before they tested for histopathology and PCR. E: PCR for MDV in the spleen and blood of the inoculated chickens. Pathogen MDV serotype 1 was detected with bands of 302bp and/or 434bp.

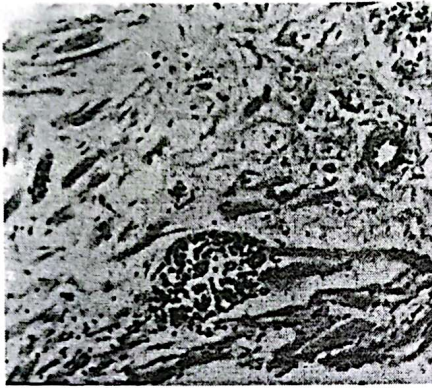
Plate (1)



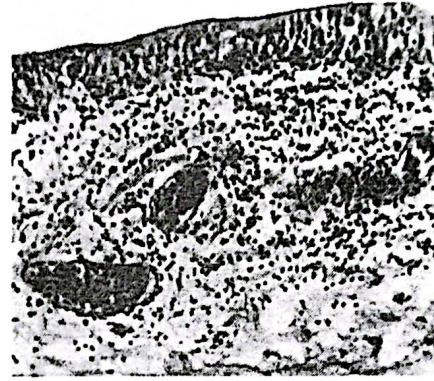
A



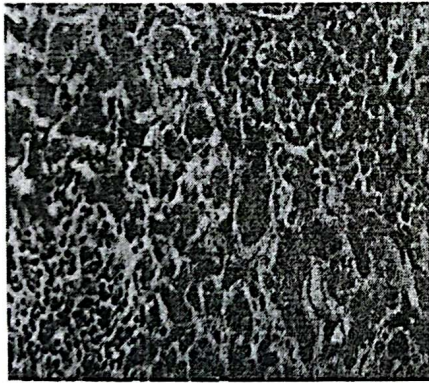
B



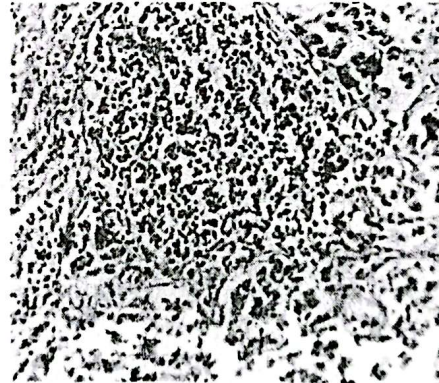
C



D



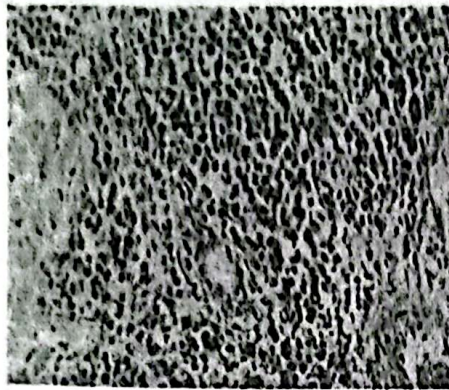
E



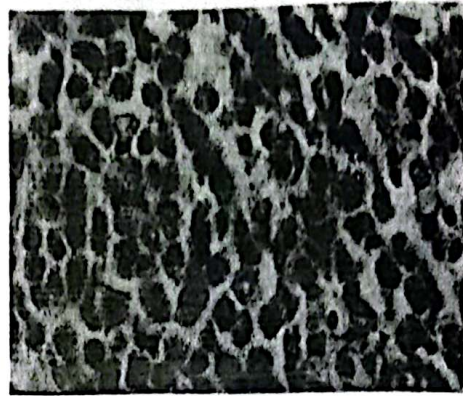
F

Plate 1: A , B and C: Eye lesions in group of chicks MDV-vaccinated and challenged with homogenate after 10 days post inoculation: oedema of the iris with focal aggregation of pleomorphic lymphocytes (H & E X100 and X400). D: Submucosal pleomorphic lymphocytes infiltration and hyperemic blood vessels in the conjunctiva. E: Liver showing focal aggregated areas of small lymphocytes and myelocytes (H & 400X). F: Proventriculus showing glandular pleomorphic lymphocytic aggregation (H & E 250).

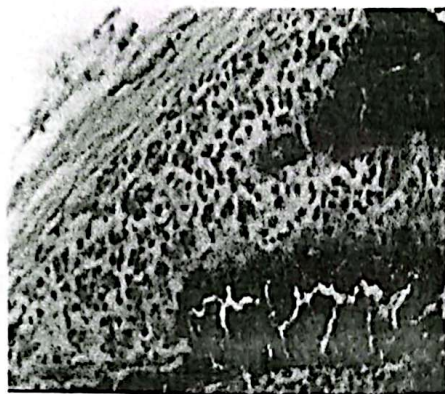
Plate (2)



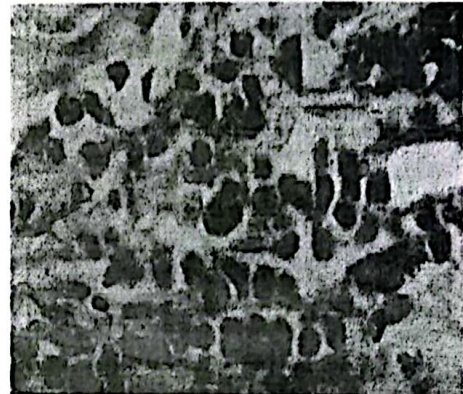
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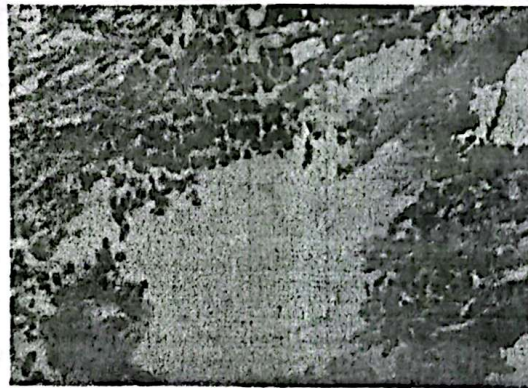
B



C



D



E

Plate 2: A and B: Liver of the chicks (MDV non-vaccinated and challenged with the homogenates) showing perivascular infiltration of myelocytes (H & E X 400 and 1000). C, D and E: Eye lesions showing the myelocytes infiltration in the ciliary processes (H & E X400 and X1000).

Plate (3)

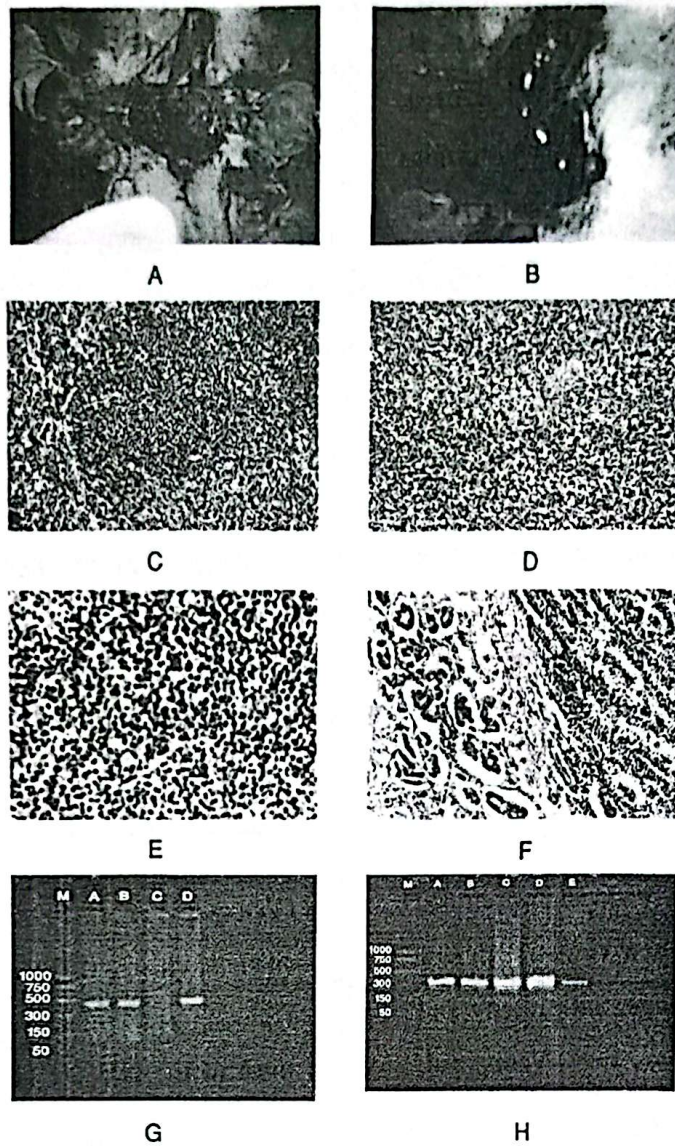


Plate 3: A and B: Thirty weeks old female chickens with visceral tumors in liver and spleen. C and E: Liver focal an predominant lymphoblast cells replacing the hepatic tissue in focal circumscribed round manner. D: Spleen showing focal necrotic areas with massive numbers of infiltrated myelocytes and erythrocytes in the pulp. F: Proventriculus showing lymphoid cells infiltration in the lamina propria with necrosis of the glandular structure. G: PCR for MDV, pathogenic MDV serotype 1 was detected in spleen tumors of the chicken (434bp; lanes A,B and D or 366bp in lane C of the photo). H: PCR universal for all ALV subgroups A, B, C, D and E; 360bp bands in spleen (lanes A to D) and blood (lane E).

tions are not usually associated with tumors and this confirms the decreased cases of tumors noticed in the last 2 years. The present study raises the possibility that the current circulating ALV-Js are variants and their histopathological behaviour is also changeable. Employing PCR on organs in the study confirmed mixed MDV (by PCR and histopathology) and ALV (by ALV universal PCR, histopathology and serology for ALV-J) infection. Based on the histopathological lesions seen in the examined organs, it is likely that the virus in both cases is a ALV-J. PCR conducted in the study could not detect any of these viruses. Hence, such negativity in PCR and the presence of ALV-J antibodies in sera of the affected chickens highly suggest that the ALV in such cases is a variant ALV-J. The susceptibility of cultured monocytes and bone marrow cells from different chicken lines for in vitro transformation by an acute transforming variant strain of ALV-J HPRS-103 (966) showed a correlation with the in vivo susceptibility to myeloid leucosis and other tumors (Arshad et al., 1997). The mortalities from tumors seen in the field suggested that these chickens generated from infected breeding stocks (Venugopal, 1999). Many factors play a role in the existence of ALV-J variants strains. First, the congenital and horizontal transmission of the virus. Second, the environment which always modified and may enhance the expression and shedding of ALV-J infections. Such stress factors, specially high bird density, excessive or rough

handling, improper beak trimming, deficiencies in feeder or drinker space availability, inadequate male/female ratios, vaccine immunosuppression, vaccine overload, concomitant immunosuppressive disease and nutritional deficiencies or imbalance, can contribute in the enhancement of the ALV-J genes expression and possible shedding of viruses (Zavala, 2000). Also, MDV, IBD, CAV are synergetic with ALV-J (Zavala, 2000). It is not clear in the cases of mixed infection reported in the current study that the primary infection of the chicks under study in both cases (with tumor or experimentally inoculated chicks) was with ALV-J followed by MDV or MDV followed by ALV. The transmission of all exogenous ALV occurs either by vertical or horizontal and the ALV infection status is expressed by the absence (-) or presence (+) of viremia (V), serum antibodies (A) and virus shedding (S). In case of vertical transmission, the chicks (infected from hens) became immunotolerant and express V+ A- S+. These chicks more likely develop tumors. These chicks and their meconium in hatches are the source of virus horizontal transmission. Horizontal transmission post hatch with only ALV-J differs in its outcome. As in case of ALV subgroup other than J, the outcoming chicks considered immuno non-shedders (V- A+ S-). In case ALV-J, the resulted chicks from the horizontal transmission either immunotolerant (V+ A- S+ or immune status with transient viremia. Some of these birds may also shed (V- A+ S+). The

erance in some of these birds are not complete and plasma cells can be detected in their spleen in spite of the absence of neutralizing antibodies in the serum of these chicks (Russel et al., 1997). In the present study, plasma cells were seen in the spleen during the histopathology examination of the organs in both cases. It is not clear if these cells are due to ALV-J infection or any other pathogens. Even more, the factors that determine the tolerance or immune status of ALV-J are so many and not clearly understood (Venugopal, 1999). The expression of EVA-HP which constitutes the genetic backbone of the ALV-J env sequences may play a role in postulating the induction of tolerant status (Smith et al., 1999). The presence of anti-ALV-J antibodies in sera collected from the birds in the current study may be attributed to change in the immune status of the ALV-J infected birds and these changes may confirm that these viruses are variant ALV-Js.

Changing behaviour was seen before with the increase occurrence of ALV-J variants with sequence changes in variable regions specially in flocks undergoing eradication programmes (Venugopal et al., 1998, Venugopal, 1999).

In conclusion, the study reports the existence of mixed infection of MDV and ALV in the examined chicks. The negativity of the employed PCR for ALV-J and the positivity of these samples in histopathology and serology for ALV-J indicate

the need for continuous update the primers used in the specific PCR and the urgent combination between PCR, virus isolation and histopathology in the diagnosis of ALV-J cases. The histopathology behaviour of the detected ALV-J and lesions seen in eye represent new finding and may indicate the presence of circulating variant viruses.

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