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Visceral lymphomas due to co-infection of Marek's disease virus- avian leukosis virus A–E in Japanese silkie fowl

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Abstract

Increased mortality was noticed in an 8-month-old farm of white silkie fowl (*Gallus gallus domesticus*) during March 2013. Three birds were necropsied and investigated to determine the etiology. Splenomegaly, hepatomegaly, and enlarged kidneys, testes, and pancreas were observed. Surface mottling or white-tan nodules (5–10 mm) were observed on parenchymatous organs. Peritoneal thickening was noticed in two cases. Lymphomatous lesions comprising diffusely proliferating small-to-medium lymphocytes and large lymophoblasts in some areas and multicentric foci of large lymphoblastic cells almost of the same size were observed in most of the visceral organs in all three birds including spleen, liver, kidneys, heart, lungs, testes, and ovaries. Immunolabeling with Marek's disease virus (MDV) pp38 antibody identified the pp38 antigen in lymphocytes within splenic lymphomatous foci. The lymphomatous foci in spleen, liver, lungs, kidneys, and intestine were positive for CD3 (T-cell marker), and negative for CD20 (B-cell marker) and Pax5 (B-cell transcription factor). PCR amplification of the 132 base pair tandem repeat (BamH1-H, D fragments) of MDV and gp85-env gene of avian leucosis virus subgroups A–E (ALV A–E) in formalin-fixed paraffin-embedded tissues of lymphomas in all three birds yielded 434 and 300 bp amplicons, respectively. Visceral lymphomas due to MDV-ALV A–E co-infection are reported for the first time in Japanese silkie fowl.

Key Words: Marek's disease, MDV, ALV, Japanese silkie, Lymphoma, IHC, pp38, CD3, BamH1-H, gp85-env.

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Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by the Marek's disease virus (MDV), or Gallid Alphaherpesvirus 2 (GaHV2); this cell-associated oncogenic serotype 1 is a member of genus *Mardivirus* within the family Herpesviridae, subfamily Alphaherpesvirinae[32]. There are three MDV serotypes, GaHV-2 or MDV serotype 1 (MDV-1), GaHV-3 or MDV-2, and *Meleagrid herpesvirus 1* or MDV-3 (herpesvirus of turkeys)[32]. Administration of live vaccines currently protect against MD, but MDV-1 become more virulent[38], as several MD cases have been reported in vaccinated farms[13,15]. MDV-1 infection has been reported in several *Galliformes* genera; domestic chickens are the most important natural host[7]. All chicken breeds and species, including game fowl, native breeds, and jungle fowl, are susceptible to MDV infection and tumor development. Quail, turkey, and pheasant are also susceptible to viral infection and disease[32]. Ducks, geese, sparrows, partridge, pigeons, and peafowl[2,7,18] are likely resistant to infection, but notably, ducks inoculated with MDV developed antibodies[2]. Recently, MD was reported in a white-fronted goose (*Anser albifrons*) that migrated from Russia to Hokkaido, Japan[23]. The MDV-1 genome was later found in goose and duck feather tips throughout Hokkaido, but it was not detected in other wild waterfowl[24]. MDV (JS-1, JS-2 and JS-3) has been isolated from black and red face Japanese silkies (*Gallus gallus*). Gross MD lesions was not developed when the virus was inoculated into WSU-VS chickens; a highly MD-susceptible experimental line of White Leghorn, but minimal lymphocytic infiltration of C-type MD lesions; characterized by light infiltration by plasma cells and small lymphocytes, was observed in nerves[7].

Avian leukosis viruses (ALVs) are a group of oncogenetic retroviruses that cause leukosis / sarcoma neoplasia and production problems in chickens[25]. ALVs are the most common avian retroviruses in the field. ALV was classified into six subgroups (A–E and J) in chickens according to their host range, antibody neutralization and receptor interference[25]. Subgroups A and B viruses induce lymphoid leukosis. Subgroups C and D have rarely been reported in the field[31]. Subgroup E is an endogenous leukosis virus with no pathogenicity[8]. Subgroup J (ALV-J) was first reported in the UK[28] and recently a link between ALV-J and myeloid leukosis and other tumors in meat-type and layer-type chickens has been illustrated[6,39]. In the present case series, an outbreak of MD lymphoma was detected in the visceral organs of Japanese silkie fowl, and the pathological, immunohistochemical, and molecular findings are described.

Materials and methods

Case history: At an 8-month-old farm comprising 800 white silkie fowl (*Gallus gallus domesticus*), mortality increased during March 2013. Three dead birds were sent to the laboratory of Veterinary Pathology, Gifu University for necropsy and further etiologic investigation. No vaccination protocol against MDV was applied to the farm before the incidence of mortalities.

Histopathological Examinations: Sections of lung, intestine, liver, heart, spleen, kidney, proventriculus, and gizzard from the 3 birds were fixed in 10% formalin solution, processed routinely, and embedded in paraffin wax. Sections 2–4 μm thick were cut and stained with either hematoxylin and eosin (HE) stain or immunohistochemistry.

Immunohistochemistry: Paraffin-embedded sections of spleen, liver, and kidney from the birds were submitted to Iwate University for MDV-1 immunohistochemical staining. The avidin-biotin peroxidase complex (ABC) method for immunoperoxidase staining was performed to detect pp38 antigen. Antigen was retrieved using ImmuneActive Retrieval Solution at 121°C for

Table 1. Primers used for detection of MDV, REV and ALV

	Primers	Size	
MDV	F:5'TACTTCCTATATAGATTG AGA CGT 3' R: 5'GAGATCCTCGTAAGGTGTAATATA 3'	434 bp/BamH1-H2 gene	(Bradley *et al.*, 1989)
REV (SNV-LTR)	F: AATGGTTGTAAAGGGCAGAT R: CTCCTCTCACTGCCAATCT	200 bp/REV-LTR	(Kim *et al.*, 2011)
ALV-A-E	H5(F): GGATGAGGTGACTAAGAAAG AD1(R): GGGAGGTGGCTGACTGTGT	295–326 bp	(Smith *et al.*, 1998)
ALV-J	H5-GGATGAGGTGACTAAGAAAG R11-TGGGGGTGGGAAGGGAGGGT	445/upstream of the pol region to gp85	(Silva *et al.*, 2000)

15 minutes. An MDV-1-specific mouse monoclonal antibody H19 (pp38) (kindly provided by Dr. Lucy Lee, ADOL, East Lansing, MI) was applied at a 1 : 1,000 concentration for 10 minutes. Sections were counterstained with hematoxylin. Paraffin sections of known MDV-1 positive tissues (skin) were included as a positive control; to generate a negative control, the primary antibody was replaced with buffer. Separate paraffin-embedded spleen, liver, lung, small intestine, and heart sections were immunostained separately using rabbit whole antiserum with reactivity to human T cell CD3 peptide (Sigma-Aldrich, USA) diluted 1 : 200 using Dako Real™ Antibody Diluent Buffer (Dakocytomation, Dako, Glostrup, Denmark), CD20 (rabbit polyclonal, prediluted; Acris Antibodies, Inc., San Diego, CA, USA) and Pax5 (rabbit polyclonal, prediluted; Abcam, Cambridge, MA, USA)[4,37]. The anti-human antibodies have tested cross-reactivity to chicken molecules. Other specific anti-chicken antibodies required frozen tissues that were not available in the present cases. Antigen was retrieved by steaming the specimens in antigen retrieval solution (Dako, Glostrup, Denmark) for 15 minutes at 121°C. The specimens were then immunolabeled using the Dako REAL Envision Detection System Peroxidase/DAB+, Rabbit detection system (Dako, Glostrup, Denmark) according to the manufacturer's instructions.

DNA extraction: DNA was extracted from the formalin-fixed paraffin embedded (FFPE) lymphoma specimens isolated obtained from various organs including spleen, liver, kidney, and ovary using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The paraffin was removed from the 5 tissue sections (5-μm thick) using xylene, and the resulting tissue pellets were washed with 1 mL of 100% ethanol. The micro-centrifuge tubes were left open at room temperature for 30 minutes, which allowed the residual ethanol to evaporate. The specimens were re-suspended in animal tissue lysis buffer (180 μL) and proteinase K (20 μL), mixed by vortexing, and incubated overnight at 56°C to allow complete tissue digestion. The digested mixture was loaded into the elution column, washed twice with buffer, and the DNA was isolated using elution buffer. The eluted DNA was stored at −20°C until use.

PCR detection of MDV, reticuloendotheliosis virus (REV) and ALV Subgroups A to E: The PCR was based on a primer flanking 132 bp repeat sequence located within the BamH1-H fragment in MDV-1 DNA[10], REV-LTR[19], H5/R11[33] amplifying the *env* gene of ALV-J and H5/AD1 for detection of ALV subgroups A–E[34] (Table 1). PCR was performed at a 50 μL final reaction volume in thin-walled capped PCR tubes containing 2 μL of template DNA, 5 μL of PCR buffer (TaKaRa Ex Taq™ Polymerase kit), 25 mM MgCl2, 4 μL of dNTPs, 1 μL of each primer (Sigma-Aldrich, USA), and 1 U Taq DNA polymerase (TaKaRa Ex Taq™ Polymerase kit, Tokyo, Japan). PCR was performed in a thermal cycler (Takara Bio Inc., Shiga,

Table 2. PCR conditions used for amplification of MDV, REV and ALV

Step	MDV-(BamH1-H2)			REV-(SNV-LTR)			ALV-J-(H5/R11)			ALV-A-E (H5-AD1)		
	°C	Time	No. of cycles	°C	Time	No. of cycles	°C	Time	No. of cycles	°C	Time	No. of cycles
Initial denaturation	94°C	1 min	1	95°C	3 min	1	95°C	3 min	1	94°C	4 min	1
Denaturation	91°C	1 min	31	95°C	40 sec	36	95°C	1 min	29	94°C	1 min	30
Anneling	55°C	10 sec		57°C	40 sec		57°C	1 min		58°C	1 min	
Extension	72°C	1 min		72°C	30 sec		72°C	30 sec		72°C	150 sec	
Post-PCR extension	72°C	10 min	1	72°C	8 min	1	72°C	5 min	1	72°C	10 min	1
Post-run	4°C			4°C			4°C			4°C		

Japan) and PCR conditions are demonstrated in Table 2. After the amplification, 5 μL of the reaction mixture was electrophoresed in a 2% agarose gel, stained with ethidium bromide, and visualized using a Gel documentation system (SynGene, Gene Genius Bio Imaging System, UK).

Results

Cases history and clinical signs

An 8-month-old farm of 800 white silkie fowls (*Gallus gallus domesticus*) showed increased mortality during March 2013. The deceased birds were emaciated with soft droppings. The morbidity and mortality rates were 32% and 31%, respectively.

Gross pathology

In case No. 1, the pancreas and testis were enlarged (Fig. 1a), and the peritoneum was thickened, dark, and contained minute foci. In case No. 2, the liver was enlarged, had rounded borders, and appeared mottled (Fig. 1b). White-tan nodules were present on the hepatic parietal surface. Splenomegaly with surface mottling was observed (Fig. 1c), as well as renal and testicular enlargement. Misshapen ova, ovarian distortion, and oviduct atrophy were observed in Case No. 3. Moreover, splenomegaly and hepatomegaly with variably sized (5 mm−10 mm) multiple raised gray-white surface nodules were present (Fig. 1d). The peritoneum was thickened in 2 cases.

Histopathology

Lymphomatous lesions were observed in most of the visceral organs of all three cases including the spleen, liver, kidneys, heart, lungs, testes, and ovaries. The cellular infiltrates comprised diffusely proliferating small-to-medium lymphocytes and large lymphoblasts (Fig. 2a). The tumor cellular composition was similar in different organs and tissues. The visceral neoplastic lesions were categorized microscopically into three grades by two pathologists independently as follows: Mild: normal organ architecture mostly intact, with two to three small lymphoid foci per section primarily comprising lymphocytes; Moderate: neoplastic lymphoid cell proliferation and a higher number and size of lymphoid foci with two or more foci potentially coalescing into large areas of destruction; and Severe: massive cellular infiltration, numerous mitotic figures, cellular pleomorphism, and nuclear hyperchromasia, along with grossly visibly white neoplastic foci of varying sizes from minute to massive. The distribution of these lesion categories in the three cases is summarized in Table 3.

The spleen contained extensive lymphomatous infiltration in the periarterial, periellipsoidal lymphoid sheathes, and perivenular tissue, leaving a compressed red pulp (Fig. 2b). In the liver, lymphomatous infiltration was present

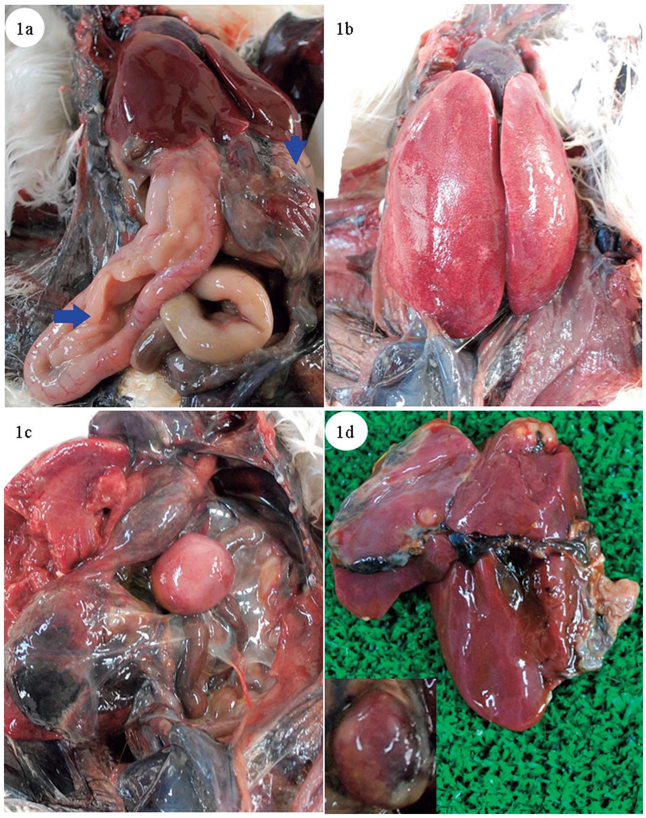

Fig. 1. Gross lesions of spontaneous visceral lymphomas in Japanese silkie fowls; 1a: Enlargement of pancreas (thick arrow) and thickening of the peritoneum (thin arrow) was observed in case No. 1. 1b: Hepatomegaly and (1c) splenomegaly with mottled surfaces were observed in case No. 2. 1d: Hepatomegaly and splenomegaly (inset) with grayish white nodules of varied sizes (5 mm–10 mm) was observed in case No. 3.

within the portal triads and greatly coalesced with neighboring nodules to form extensive lymphoma lesions (Fig. 2c). Although most of neoplastic foci composed of varied size lymphoid cells, there was clonal proliferation of almost equal-sized lymphoblasts similar to ALV lymphoma in some foci. Lymphomatous foci consisted of aggregates of large lymphoid cells (lymphoblasts) that are similar in size and early developmental stage with poorly defined basophilic cytoplasmic and vesicular nuclei with marginated chromatin and conspicuous acidophilic nucleoli (Fig. 2d). Significant lymphomatous tissue was present in the pancreas of case No. 1. The acini were dispersed by the massive lymphoblastic infiltration (Fig. 2d). Minute lymphocytic foci were observed in the pericardium. The tertiary respiratory bronchioles were thickened, and extensive lymphomatous infiltration obliterated the airway in 2 birds. Multifocal lymphomatous renal infiltration was observed in all three birds. In the gastrointestinal tract, extensive lymphomatous tissue was observed throughout the entire intestinal wall, proventricular glands, and the muscular and glandular stomach serosa. The ovary and testis were infiltrated by lymphomatous tissue in one bird each. The bursa of Fabricius contained interfollicular lymphocytic infiltration in one bird, while the structure was normal in the other 2 birds.

Immunohistochemistry

Phosphoprotein 38 (pp38) antigen of MDV-1 was present within the lymphocytes of splenic lymphomatous foci (Fig. 3a) and in a few cells within the hepatic lymphoid foci. Feather follicles from chickens inoculated with Md/5 strain of MDV-1 were positively stained with pp38

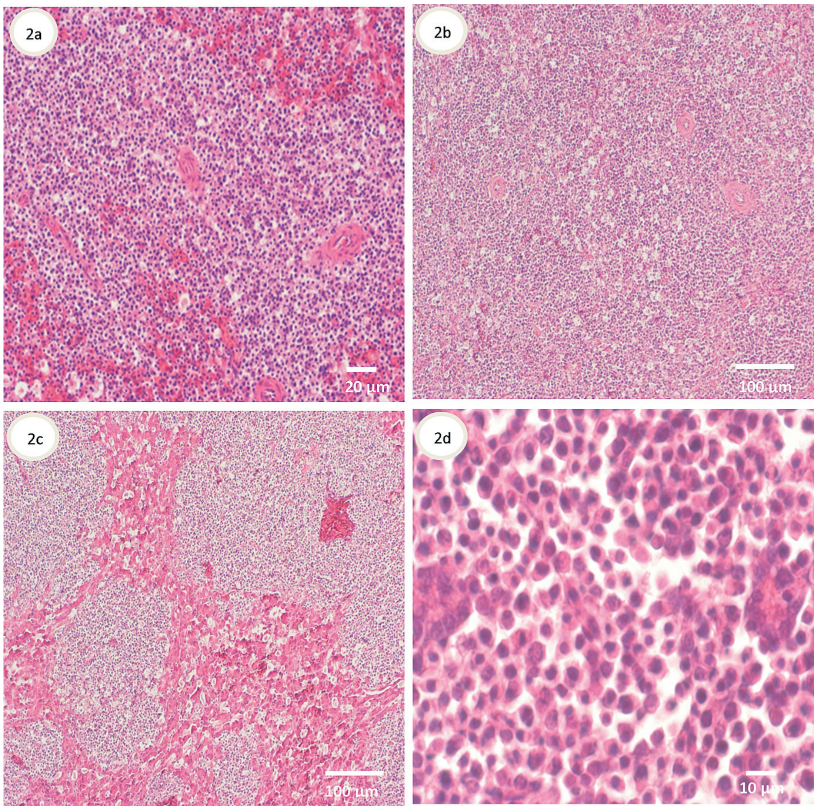

Fig. 2. Histopathological lesions of spontaneous visceral lymphomas in Japanese silkie fowls; 2a: Lymphomatous lesions composed of diffusely proliferating small-to- medium lymphocytes and large lymphoblast in spleen (case 2), HE. 2b: Extensive lymphomatous infiltrations in the periarterial and periellipsoidal lymphoid sheathes and perivenular tissue leaving a compressed narrow red pulp (case 3), HE. 2c: Multifocal lymphomatous infiltration in hepatic tissue and portal triad that coalesced with neighboring nodule forming extensive area of lymphomas (case 2), HE. 2d: Severe lymphomatous infiltration in pancreas dispersing pancreatic acini (case 1), HE.

Table 3. The distribution of lymphomatous lesions in the cases of Japanese silkie fowls

Case No. (sex)	Liver	Spleen	Heart	lungs	kidneys	Proventriculus	Gizzard	Intestine/ serosa	pancreas	Ovaries/ testis	Brain
1 (♂)	++	++	+	+++	+++	+++	++	+++	+++	+++	−
2 (♂)	+++	+++	+	++	+++	++	++	+++	−	−	nd
3 (♀)	+++	++	+	+	++	+	++	+++	++	++	nd

+, mild; ++, moderate; +++, severe; nd, not done.

antibodies and served as control positive (Fig. 3b). The lymphocytes contained in lymphomatous foci within the spleen, liver, lungs, kidneys, and intestine were positively stained with CD3 antibody in all affected fowls (Fig. 3c). In contrast, the staining with B-cell markers, CD20 and Pax5, were negative in the lymphomatous tissues (Fig. 3d).

Polymerase chain reaction

The PCR amplification of the 132 base pair tandem repeat (BamH1-H, D fragments) of MDV-1 produced amplicons of the expected size (434 bp), which were isolated from lymphoma-containing FFPE splenic, hepatic and renal tissues in all three birds (Fig. 4a). Amplification of the gp85-env gene of ALV A–E in FFPE splenic, hepatic and renal lymphomas in all three birds yielded ~ 300 bp amplicons (Fig. 4b). Ovaries of

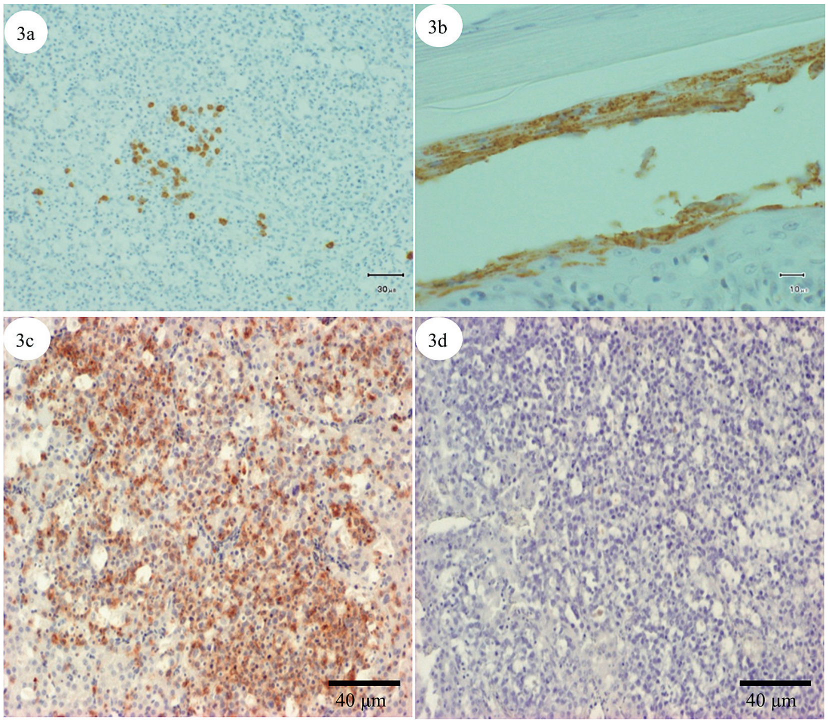

Fig. 3. 3a. Immunolabeling with an antibody for the pp38 protein of MDV-1 detected pp38 antigen in lymphocytes of lymphomatous foci in spleen. ABC immunoperoxidase technique, counterstain Mayer's hematoxylin. **3b: Positive control of feather follicle from chickens inoculated with Md/5 strain of MDV-1.** ABC immunoperoxidase technique, counterstain Mayer's hematoxylin. **3c: Lymphomatous foci of liver were CD3 positive. 3d: Lymphomatous foci of liver were CD20 negative.** LSABC immunoperoxidase technique, counterstain Mayer's hematoxylin.

chicken 3 were also positive for MDV-1 and ALV A–E. Amplification of REV-LTR gene for detection of REV and H5/R11 for detection of the env gene of ALV-J failed to yield any bands specific for these viruses.

Discussion

The gross lesions and histological features in the present cases were consistent with lymphoma. The positive immunostaining of the neoplastic cells by anti-human CD3 T-cell antibodies and MDV pp38 positivity supported the diagnosis of MD. Moreover, amplification of the 434 bp region within the MDV BamH/H gene confirmed the MD diagnosis. Although a negative immunostaining of B-lymphocytes with B-cell human markers, the histopathological findings of clonal foci (homogenous population) of lymphoblast and amplification of gp85-env gene specific for exogenous ALV subgroups A–E in lymphoma confirmed the co-infection of birds with MDV-1 and ALV A–E. The Japanese Silkie (Ukokkei) was introduced from China or India early in the 17th century; the modern Silkie breeds are thought to have been established in China and Japan[29]. MDV-1 has not been isolated from the Japanese silkie, however apathogenic MDV-2 isolates resembling the HN strain were isolated from captive Japanese silkies, red jungle fowl, and Ceylon jungle fowl[7]. Lymphomas involving mainly the skin have been reported in farms of Chinese silkies due to MDV-1[20]. In the present report, no vaccination protocol against MDV-1 was applied to the farm before the incidence of MDV-1-ALV A–E coinfection. The yard of the farm is completely isolated from surrounding environment; therefore the source of infection could not be confirmed. Previous reports

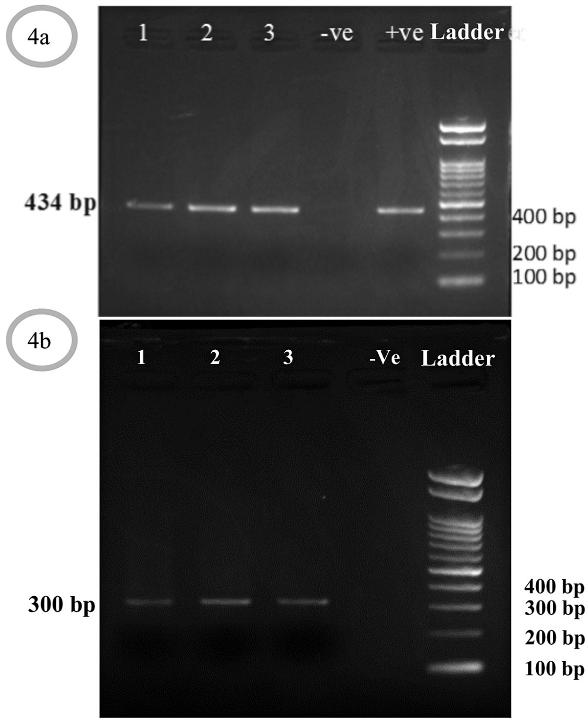

Fig. 4. 4a. Amplification of the 132 base pair tandem repeat (BamH1-H, D fragments) in FFPE tissues containing lymphomas in the 3 birds yield amplicons of 434 bp. 4b. Amplification of the gp85-env gene in FFPE lymphomas in all three birds yielded ~ 300 bp amplicons.

of ALV and REV contamination to MDV[1,40], fowl pox[11], yellow fever[16], measles, mumps and rubella[17] vaccines have been existed. In the current cases no protocol for MDV vaccination was applied.

Visceral lymphoid tumors in birds 16 weeks or older lacking neoplastic involvement of the bursa of Fabricius indicates MDV-1 and/or REV infection. Moreover, a mixed population of small to large lymphocytes, lymphoblasts, plasma cells, and macrophages are typically found in MD tumors and affected nerves[32]. MD tumor lesions comprise pleomorphic lymphoid cells that predominately express CD4/CD8 markers and minimally express IgM or B-cell markers, if at all[14,32]. Other nodular lesions were observed on liver and it consisted of aggregates of large lymphoid cells (lymphoblasts) that are similar in size and early developmental stage with poorly defined basophilic cytoplasmic and vesicular nuclei with marginated chromatin and conspicuous acidophilic nucleoli. These lymphoblastic lesions are similar to findings of lymphoid leukosis[25]. In the present study, frozen tissues were not available to perform CD4/CD8, CD79a immunostaining. Therefore, immunohistochemistry was performed on the FFPE tissues using an anti-human CD3 antibody and anti-CD20 and anti-Pax5 rabbit polyclonal antibodies which were previously used to immunostain T and B cells in wild birds, respectively[4,27,37]. Most of lymphomatous cells were T-lymphocytes with no or minimal B-lymphocytes. The antibodies used for CD20 and Pax5 may not cross react with avian molecules[37]. In the present study, anti-pp38 MDV-1 was positively immunostained in some neoplastic cells within splenic lymphoma lesions[26]. Molecular detection of MDV-1 DNA in tumors, especially neoplastic nodules consistent with gross and histopathological characteristics, is sufficient for MD diagnosis[10]. Amplification of the MDV-1 132 base pair tandem repeat (BamH1-H, D fragments) produced the expected amplicons (434bp) in FFPE tissues containing lymphomas. This gene has been amplified from whole virus culture or feather tips for MDV-1 diagnosis, but it has not yet been amplified from FFPE tissue[9,10]. The association of MDV-1 with tumor cells is confirmatory. Similarly, the histopathological findings of lymphoid foci with large lymphoblast with marginated chromatin nuclei and amplification of gp85-env gene of ALV A–E at ~ 300 bp amplicon in lymphoma confirmed the co-infection of birds with MDV-1 and ALV- A–E[41].

Formaldehyde tissue fixation leads to tissue cross-linking, which causes the DNA and RNA to fragment and decrease in quality[22]. The DNA and RNA extraction methods from FFPE tissues requires several modification to optimize nucleic acid yield and size; despite this, PCR amplification of products above 300–400 bp remains limited[12,22]. In the present study, PCR efficiently amplified the 434 bp product, possibly due to the short storage duration of the specimens, as our laboratory regularly processes samples within nearly one week following necropsy. Recently, MDV-1 and REV were diagnosed molecularly from FFPE tissues by targeting the pp38 and LTR genes, respectively, producing amplicons smaller than 250 bp[5]. Avian and human viral detection from FFPE tissues has been increasingly implemented[21,30,35,36]. PCR of FFPE tissues in addition to conventional methods such as necropsy, histopathology, and immunohistochemistry is appropriate for diagnosis of avian viral infections.

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