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Marek's Disease in Backyard Chickens, A Study of Pathologic Findings and Viral Loads in Tumorous and Nontumorous Birds

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SUMMARY. Marek's disease (MD) is a major cause of mortality in backyard chickens. The diagnosis of MD is complex, however, and knowledge of Marek's disease virus (MDV) in spontaneous field cases such as in backyard chickens is largely unknown. In this study, 40 backyard chickens with a presumptive MD diagnosis based on histologic lymphoid infiltrations in peripheral nerves with and without lymphomas were investigated. Twenty-eight of the birds were submitted to the diagnostic laboratory for disease explorations, and 12 chickens were from a flock in which some members demonstrated anisocoria and pupil irregularities compatible with ocular MD. Histologic scores were established for brain, peripheral nerves, heart, lung, liver, kidney, and gonad sections, ranging from mild (+) to severe (+++) lymphoid infiltrations. Twelve chickens had gross lymphomas, and all but two chickens had mild to severe peripheral nerve lymphoid infiltrates. There were no age or breed predispositions in the study group. Quantification of serotypes MDV-1, –2, and –3 performed with real-time PCR demonstrated high correlation ($R^2 = 0.94$) between fresh and fixed spleen specimens, as well as between histopathology scores and MDV-1 viral loads. MDV-2 DNA was detected in a portion of the chickens, likely consistent with naturally occurring virus, whereas the vaccine strain MDV-3 was rarely detected. Significant differences in MDV-1 viral loads between tumorous and nontumorous chickens were observed, in which a ratio of MDV-1 glycoprotein B/glyceraldehyde-3-phosphate dehydrogenase ≥ 0.5 was suggestive of gross tumors in this study. We propose that real-time PCR may be a good tool for MD diagnosis in backyard chickens.

RESUMEN. Enfermedad de Marek en pollos de traspatio, un estudio de hallazgos patológicos y de cargas virales en aves con o sin tumores.

La enfermedad de Marek (MD) es una causa importante de mortalidad en pollos de traspatio. El diagnóstico de la enfermedad de Marek es complejo, sin embargo, el conocimiento de virus de la enfermedad de Marek (MDV) en los casos espontáneos de pollos de traspatio es en gran parte desconocido. En este estudio, se investigaron 40 gallinas de traspatio con un diagnóstico presuntivo de enfermedad de Marek con base en los infiltrados linfocitarios histológicos en los nervios periféricos con y sin linfomas. Veintiocho de las aves fueron enviadas al laboratorio de diagnóstico para estudio de enfermedad y doce pollos eran de una parvada en la que algunas aves manifestaron anisocoria e irregularidades de la pupila compatibles con la enfermedad de Marek ocular. Se establecieron puntuaciones histológicas para cortes de cerebro, nervios periféricos, corazón, pulmón, hígado, riñón, y gónadas, que fueron de infiltrados linfocitarios leves (+) a severos (+++). Doce pollos mostraron linfomas macroscópicos y todos los pollos con excepción de dos, mostraron infiltrados linfocitarios de leves a severos en los nervios periféricos. No hubo predisposición por edad o por raza en el grupo de estudio. La cuantificación de los serotipos 1, 2 y 3 de la enfermedad de Marek realizada mediante PCR en tiempo real demostró una alta correlación ($R^2 = 0.94$) entre las muestras de bazo frescas y fijadas, así como entre las puntuaciones de la histopatología y las cargas virales de los serotipos 1 y 2. Se detectó ADN del serotipo 2 en algunos de los pollos, probablemente consistente con virus de campo, mientras que raramente se detectó la cepa vacunal del serotipo 3. Se observaron diferencias significativas en las cargas virales del serotipo 1 entre los pollos con y sin tumores, donde la relación de la glicoproteína B del serotipo 1/ gliceraldehído-3-fosfato deshidrogenasa ≥ 0.5 fue sugestiva de tumores macroscópicos en este estudio. Se propone que la PCR en tiempo real puede ser una buena herramienta para el diagnóstico de la enfermedad de Marek en aves de traspatio.

Key words: backyard chickens, Marek's disease, MDV serotypes, real-time PCR, absolute quantitation, pathology, histopathology

Abbreviations:CAHFS = California Animal Health and Food Safety; COD = cause of death; CS = clinical signs; D = spontaneous death; E = euthanasia; FFPE = formalin-fixed paraffin-embedded; G = general signs; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; gB = glycoprotein B; histo = histologic; HRP = horseradish peroxidase; HVT = herpesvirus of turkeys; MD = Marek's disease; MDV = Marek's disease virus; N = no; NA = not available; Neuro = neurologic signs; O = ocular; P = pneumoconiosis; PN = peripheral nerve; REV = reticuloendotheliosis; U = unknown; Y = yes

Marek's disease (MD) has caused significant mortality and economic losses in the poultry industry (36) and is a major cause of mortality in backyard chickens worldwide (29,34,40). A recent U.S. backyard poultry disease survey demonstrated that MD is the fifth most prevalent condition affecting chickens throughout the nation (19), and it has been shown to be the number one cause of mortality in backyard chickens in California (29). Because backyard chickens are primarily kept as pets and for hobby with lax biosecurity systems and poor vaccination strategies (13,24,27) and the causative Marek's disease virus is highly contagious and environmentally resistant (36), it is likely that MD will continue to be a significant problem in backyard chickens.

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Marek's disease virus (MDV) is a cell-associated alphaherpesvirus. Of the three serotypes, MDV-1 is the pathogenic serotype because of its oncogenic properties, whereas MDV-2 and MDV-3 (herpesvirus of turkeys; HVT) are nonpathogenic and primarily used for immunization against MDV-1 (36). The oncogenic viral pathogenesis occurs in four phases; infection on exposure to MDV-1 can occur in as early as 1-day-old chicks, in which the cytolytic phase ensues, followed by latency, second cytolytic phase, and finally the transformation of T lymphocytes and development of lymphoproliferative disease or lymphomas (6). The mechanism(s) of progression between the phases is mostly unknown, and the durations are unpredictable, in which some birds will succumb to fulminant neoplastic disease within weeks of infection, and others may not develop disease during their lifetime. The complex interaction between the host genetic line and the viral strains seems to be the two main indicators of outcome of infection (44).

Vaccination is the primary means of control of MD, and the vigorous vaccine strategies employed by the commercial poultry industry have been mostly successful in mitigating losses (31,36). Vaccines produced from serotypes MDV-2 (e.g., SB-1), HVT, and attenuated MDV-1 (e.g., CVI988; Rispens) are used singly or jointly, with the most advanced technique being *in ovo* vaccination with Rispens (16). Nevertheless, factors such as prior virus exposure, improper handling and administration of the vaccine, and the immune status of the chicken (i.e., levels of maternal antibodies, concurrent infections) contribute to vaccine failures (4,11). Moreover, emerging virulent strains (virulent pathotypes) of MDV-1 also contribute significantly to vaccine breaks (30,41), such that the most commonly used HVT vaccine does not provide adequate immunization against very virulent MDV-1 (16). Surveys (13,28,40) and personal experience in the field (A.M., M.E.P.) reveal that keepers of backyard chickens have insufficient knowledge with respect to vaccination protocols for MD. To this day, MD has been studied in chickens with known haplotypes for natural genetic resistance, most extensively in commercial poultry lines under tightly controlled experimental conditions, where, typically, a susceptible or resistant genetic chicken line, usually specific-pathogen-free, antibody-free, or both, is used with a given MDV-1 pathotype to ascertain disease progress, viral pathogenesis, and vaccine efficacy (1). Either the host lineage or the infective MDV pathotype are fully diverse and unknown in backyard chickens.

The diagnosis of MD is complex; the virus is ubiquitous, infection does not correlate with disease, and the clinical signs and lesions may overlap with other lymphoproliferative diseases of chickens, as well as the autoimmune entity peripheral neuropathy (2,43). Researchers have identified useful criteria to aid in the differential diagnosis of MD, involving the age of the chicken, gross pathology and histopathology, immunohistochemistry, and molecular techniques. However, these criteria may yet be insufficient and often need to be utilized collectively (43). A suggested definitive diagnostic tool is the quantitative real-time PCR (real-time qPCR) analysis of tumors, where the MDV load is far greater than nontumorous tissues (16). In the field, however, the diagnostician is often left to gross and histopathologic diagnosis of MD in birds of varying or unknown ages and vaccination histories with extensive overlap of lesions, and better diagnostic tools are essential. It is also becoming apparent that for the backyard chicken owner, where individual health monitoring overrides the "flock approach" in most cases, a means to diagnosing MD is highly desirable. Here, we describe the characteristics of backyard chickens submitted to the diagnostic laboratory for disease investigations with a diagnosis of presumptive MD, the development of real-time qPCR to aid in the differentiation of birds that have MD tumors from nontumorous birds, and the correlation between histopathologic scores and MDV-1 viral loads.

MATERIALS AND METHODS

Case data and gross pathologic findings. Forty backyard chickens from 26 premises submitted to the California Animal Health and Food Safety (CAHFS) laboratory in Davis, CA, between 2013 and 2015 were included in the study. Twenty-eight birds from 25 backyard flocks had either died suddenly or had clinical disease that required euthanasia and diagnostic evaluation by the pathologist ("diagnostic cases"). The remaining 12 birds came from a flock that had been living in the same pen for the whole of their lives, in which some had been experiencing mild to severe ocular lesions compatible with MD and others were submitted for evaluation of MD status ("ocular flock").

The selection of cases were based on the given presumptive MD diagnosis by histologic evidence of lymphoid infiltrations in peripheral nerves with or without gross evidence of neoplastic disease. When present, the neoplastic disease was histologically diagnosed as "lymphoproliferative disease" or "lymphoma."

The gross exam findings and tumor distribution, if present, were recorded, in addition to any mention of the size of the spleen when available from the postmortem examination reports. The age, sex, breed, clinical signs, and vaccination status information were collected when available from the submission forms and owners. The geographic locations were also recorded from the client information, and the distribution of the tumorous and nontumorous cases were mapped out using ArcGIS 10.2 (14).

Histopathology. Hematoxylin and eosin–stained, 5-μm-thick sections of formalin-fixed, paraffin-embedded (FFPE) brain, peripheral nerve, heart, lung, liver, kidney, and gonad were evaluated for the scoring of the lymphocytic infiltrations. Two to four longitudinal peripheral nerve (PN) sections were examined in each bird; the nerves included the lumbosacral plexus, ischiatic nerves, or the brachial plexus. Additional PN sections were examined when available in other sections, such as the skin ganglia, adrenal gland region, mesentery, and the gastrointestinal tract. Histologic (histo) scores were designed for the purposes of this study as follows: few, mostly perivascularly infiltrating and/or scattered lymphocytic infiltrations (+), moderate numbers of perivascular and/or multifocal collections of lymphoid cells (++), and large multifocal to coalescing sheets of lymphocytes obliterating the tissue architecture (+++). Presence of vascular lesions was recorded as (+) when observed in at least one vessel in any examined section. Diagnostic birds submitted with ocular lesions and eyes from the ocular flock of 12 birds were examined histologically as well.

Immunohistochemistry. Immunostaining with CD3 antibody was employed on lymphoproliferative lesions from all tumorous birds and on a portion of the birds that had only histologic peripheral nerve lymphocytic infiltrations. Antigen retrieval was performed in a modified citrate buffer, pH 6.1 (Dako North America, Carpinteria, CA). Sections were blocked in 10% normal horse serum (Vector Labs, Burlingame, CA). The CD3 primary antibody was obtained from Dr. Peter Moore's Leukocyte Antigen Biology Lab, UC Davis, CA (rat anti-CD3, clone 3-12) and detected with an avidin-biotin two-step detection system (anti-rat link; streptavidin–horseradish peroxidase (HRP) label; Biocare Medical, Concord, CA). Sections were counterstained in Mayer hematoxylin, and detection was visualized with peroxidase substrate (SK-4800; Vector Labs).

Viral loads; real-time PCR. Absolute quantification of MDV-1, MDV-2, and MDV-3 viral loads in fresh frozen and FFPE (fixed) spleens of all 40 chickens was carried out by real-time qPCR using methods previously described (7,11). Briefly, DNA was isolated from

Table 1. Clinical and vaccine histories and gross exam data of the 28 diagnostic chickens.[A]

Chicken[B]	Age	CS	Duration	COD	Postmortem examination gross findings	Vaccination
1	<1 yr	G,O	1 day	E	Anisocoria	U
2	2 yr	Neuro	>2 wk	E	Right ischiatic nerve may be swollen	Y (1d)
3	2 yr	U	None	D	Visceral gout	Y (1d)
4	**6 mo**	**Neuro**	**>2 wk**	**E**	**Tumors lung, kidney, bursa**	U
5	**6.5 mo**	**Neuro**	**<1 wk**	**D**	**Tumors skin, pectoral muscle, kidney, intestine, spleen**	U
6	9.5 mo	Neuro	2 days	E	No gross lesions	N
7	NA	G	3 days	D	Salpingitis/peritonitis/salpingoperitonitis	U
8	**9 mo**	**Neuro**	**2 wk**	**D**	**Tumors lung, kidney, liver, ovary, intestine; enlarged spleen**	N
9	4 mo	Neuro	1 day	E	Liver pallor, thin	N
10	6 mo	Neuro	1 day	E	Lumbosacral plexus may be swollen	N
11	13 mo	G	2 days	D	No gross lesions, emaciated	U
12	**6 mo**	**G**	**1 day**	**D**	**Tumors lung, kidney, liver, ova, intestine; enlarged spleen**	U
13	3.5 yr	G	2 days	D	Visceral gout, right kidney aplasia	U
14	**6 mo**	**Neuro**	**None**	**D**	**Tumors lung, liver, proventriculus; enlarged spleen**	N
15	**6 mo**	**Neuro**	**6 days**	**E**	**Tumors thymus, liver, ovary; enlarged spleen**	Y (1d)
16	10 mo	Neuro	4 days	E	No gross lesions	N
17	**9 mo**	**Neuro**	**5 days**	**D**	**Tumors lung, kidney, intestine; heart pale foci, enlarged spleen**	N
18	**4 mo**	**Neuro**	**2 wk**	**E**	**Tumors skin, thigh muscle, ischiatic nerve, bursa, liver, oviduct, proventriculus**	N
19	11 mo	Neuro	3 days	E	Salpingitis/peritonitis/salpingoperitonitis, mildly enlarged spleen	Y
20	U	G	4 days	E	No gross lesions	U
21	U	Neuro	5 days	E	Right lumbosacral plexus may be thick	U
22	**6 mo**	**Neuro**	**1 day**	**D**	**Tumors lung, kidney, liver, ovary, gastrointestinal tract**	Y (1d)
23	**1.5 yr**	**Neuro**	**2 wk**	**E**	**Tumors lumbosacral plexus, kidney, ovary, liver, spleen**	N
24	**1 yr**	**Neuro**	**10 days**	**E**	**Tumors lumbosacral plexus**	Y
25	3 mo	Neuro	3 days	E	Right ischiatic nerve may be swollen	U
26	9 mo	G	9 days	E	Chronic coelomitis, liver rupture, generalized pallor	U
27	2 mo	None	None	D	No gross lesions	Y (in ovo)
28	**9 mo**	**G**	**3 wk**	**D**	**Tumors skin, pectoral muscle, intestine; small spleen**	U

[A]CS = clinical signs; COD = cause of death; G = general signs; O = ocular; Neuro = neurologic signs; E = euthanasia; D = spontaneous death; U = unknown; Y = yes; N = no.

[B]The 12 chickens with disseminated tumors are shown in boldface.

fresh frozen spleen tissue and from fixed spleen tissue scrolls (2 μm × 25-μm scrolls) following deparaffinization, using the Qiagen DNeasy blood and tissue extraction kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). A multiplex real-time qPCR was performed to determine MDV glycoprotein B (gB) copies of each serotype compared with the cellular gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using specific primers and probes and amplified with the ABI 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). Primer pairs and accompanying probe included MDV-1 gB: 5′-CGGTGGCTTTTCTAGGTTCG-3′, 5′-CCAGTGGGTTCAACCGTGA-3′, and 5′-Cy3-CATTTTCGCGGCGGTTCTAGACGG-3′ BHQ-1; MDV-2 gB: 5′-CAGTCCCACCCAACCGTAAA-3′, 5′-GAGCATACCCGTCAAGCGTAA-3′, and 5′-Cy5-TGTGGAGTGACGAGGAA-3′ BHQ-2; MDV-3 gB: 5′-CGGGCCA-TAAAACGGAATT-3′, 5′-GGCAAAGTGGAAAGAGGTAACG-3′, and 5′-JOE-CTTGCCCACTCTAGCACGCAGCATT-3′ BHQ-1; GAPDH: 5′-CAACGGTGACAGCCATTCCT-3′, 5′-ATGGTCGTTCAGTGCAATGC-3′, and 5′-FAM-CCTTTGATGCGGGTGCT-3′ BHQ-1. Results were reported as the ratio of MDV gB copies per GAPDH copies, estimated using standard curves consisting of 10-fold serial dilutions of plasmids containing either MDV gB or GAPDH. Each sample was tested in triplicate.

Statistical analyses. Statistical correlations were evaluated for differences in MDV-1, MDV-2, and MDV-3 viral loads between chickens with gross lymphoproliferative tumors and those with only histopathologic lymphoid infiltrations in peripheral nerves. Statistical significance was defined as $P \leq 0.05$; the graphs were made using Prism 6 for Mac OS X (GraphPad Software Inc., La Jolla, CA). Correlation analysis (R^2) between fresh and fixed spleen viral loads were analyzed using bivariate linear fit and ANOVA.

RESULTS

Case data and gross pathologic findings. Case histories with age, clinical signs (CS), duration of CS, cause of death (COD), gross findings, and vaccine status of the 28 diagnostic birds are given in Table 1. Sixteen of the 28 birds had histologic peripheral nerve infiltrations only (nontumorous) and 12 had disseminated tumors (tumorous cases are boldface in all tables). The tumorous chickens had nodular (mostly in the liver) to diffuse (mostly the lungs), white-tan masses that bulged on cut section with varied organ distribution. Tumors involved a section of the gastrointestinal tract in the majority of the birds ($n = 8$), followed by the kidney ($n = 7$), and liver and lungs ($n = 6$ each; Figs. 1a,b). Three birds had skin tumors, and three had tumorous enlargement of peripheral nerves. Comments on the spleen were made only in the gross reports of the tumorous birds, wherein five had enlarged spleens, one had a small spleen, and two had tumors. There was suspicious thickening of peripheral nerves in chicken nos. 2, 10, 21, and 25 on gross observation records of the pathologist (A.M.).

The ages of the diagnostic cases ranged from 2 mo to 3.5 yr, whereas the tumorous chickens were mostly around 6 mo of age, and the oldest was 1.5 yr old. Ages of birds that succumbed to

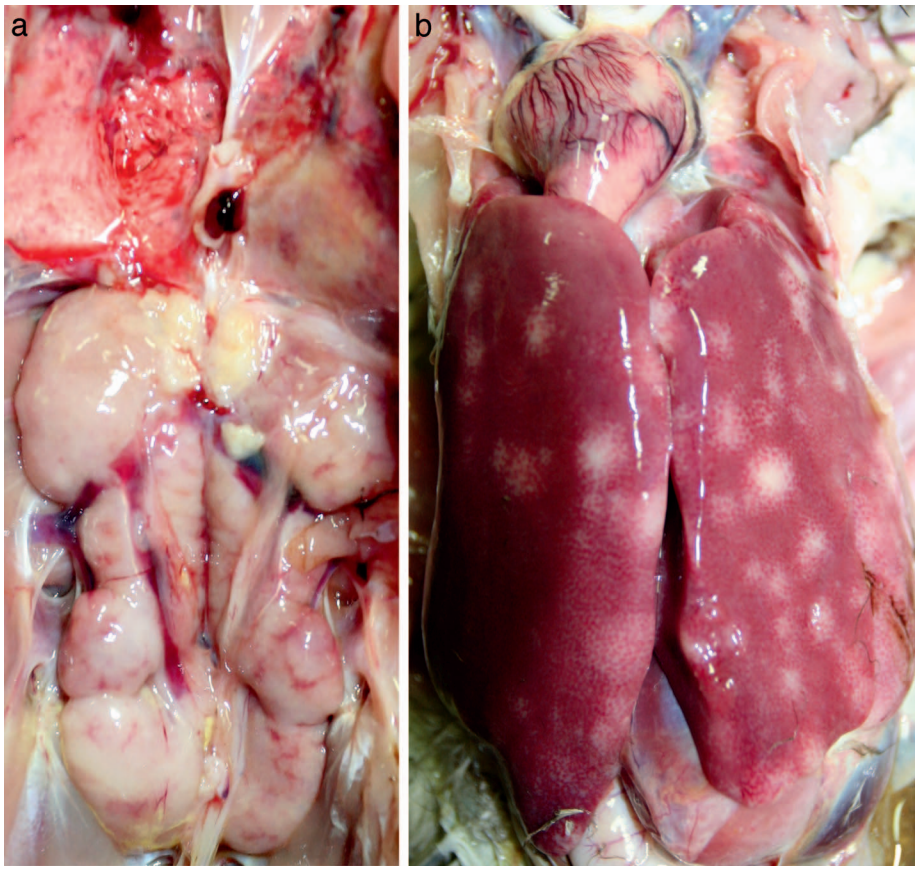

Fig. 1. MDV lymphomas in a tumorous chicken composed of nodular to diffuse white-tan bulging masses diffusely affect the kidneys and partially the lungs (a) and multifocally affect the liver and most of the heart (b).

lymphomatous tumors and those that had histologic lesions only were not different.

Eighteen of the 28 birds and all except one (no. 28) tumorous chickens showed neurologic signs compatible with leg paralysis that were described by owners as decreased ability to walk, staggering, falling, inability to stand/walk, losing balance, shaky head, progressive lameness, and curled foot. Reported general clinical signs were lethargy, unthrifty, ruffled feather, droopy, crop stasis/empty crop, and weight loss. One bird (no. 27) had no prior clinical signs and was found dead. Bird 1 had asymmetric pupil diameter (anisocoria) identified on postmortem examination, in addition to the general symptoms observed by the owner the previous day.

The COD is presented as either euthanasia or spontaneous death; 12 died suddenly or were found dead, and 16 were euthanatized at the diagnostic lab or a veterinary clinic because of clinical disease. In 16 of the 28 diagnostic cases that had no tumors, five chickens had significant gross findings that likely contributed to cause of death: visceral gout (2), in which one chicken had unilateral renal aplasia; salpingitis/peritonitis/salpingoperitonitis (2); and chronic coelomitis (1).

Of the 28 diagnostic birds, 11 had breeds recorded on the submission forms, comprising two Wyandottes, and one each of White Leghorn, Silkie, Buckeye, Golden Sex-Link, Rhode Island Red, Buff Orpington, Cornish Bantam, Old English Game Bantam, and Ameraucana. Of all studied chickens, four were male (nos. 16, 17, 21, 27).

There was no information on the vaccine status of 12 of the 28 diagnostic cases, whereas nine birds were known not to be vaccinated and seven birds were vaccinated for MD, although the owners did not know what vaccine was used. Known vaccinations were mostly done at 1 day of age ($n = 4$): one bird was vaccinated *in ovo*, and one bird at an unknown age; the unknown bird and two of the 1-day-old–vaccinated chickens were tumorous chickens. The ocular flock consisted of 2-yr-old female Rhode Island Reds in good health. These birds were vaccinated for MD in the hatchery from which they were purchased; the form of vaccine or age at vaccination are unknown.

The 12 hens in the ocular flock, six of which had ocular lesions of mild ($n = 1$) to marked ($n = 5$) anisocoria and irregular pupil shape (dyscoria), had no tumorous formations. In all, the 12 birds there had mostly subtle, regional to diffuse iris reddening and irregular surface of one or both eyes, as well as a minimal to moderately thick rim of grey discoloration of the iris circumferentially around the pupil. One chicken (no. 31) had severe unilateral grey discoloration of the iris with a few focal brown patches (anterior synechiae), as well as severe anisocoria and dyscoria. The geographic distribution of the premises of the all the cases is given in Fig. 2, demonstrating the tumorous and nontumorous birds.

Histopathology. The histologic lymphocytic infiltration scores in the brain, PNs, heart, lung, liver, kidney, and gonad ("m" for male cases) of all 40 birds are given in Table 2. Lymphoid cells predominated all lesions greatly, whereas the cell types varied remarkably from small numbers of mature, small, hyperchromatic lymphocytes to mostly blast cells, sometimes containing large, irregularly nucleated cells. A histologic score of (+) typically depicted the occasionally scattered, mostly uniform small lymphocytic

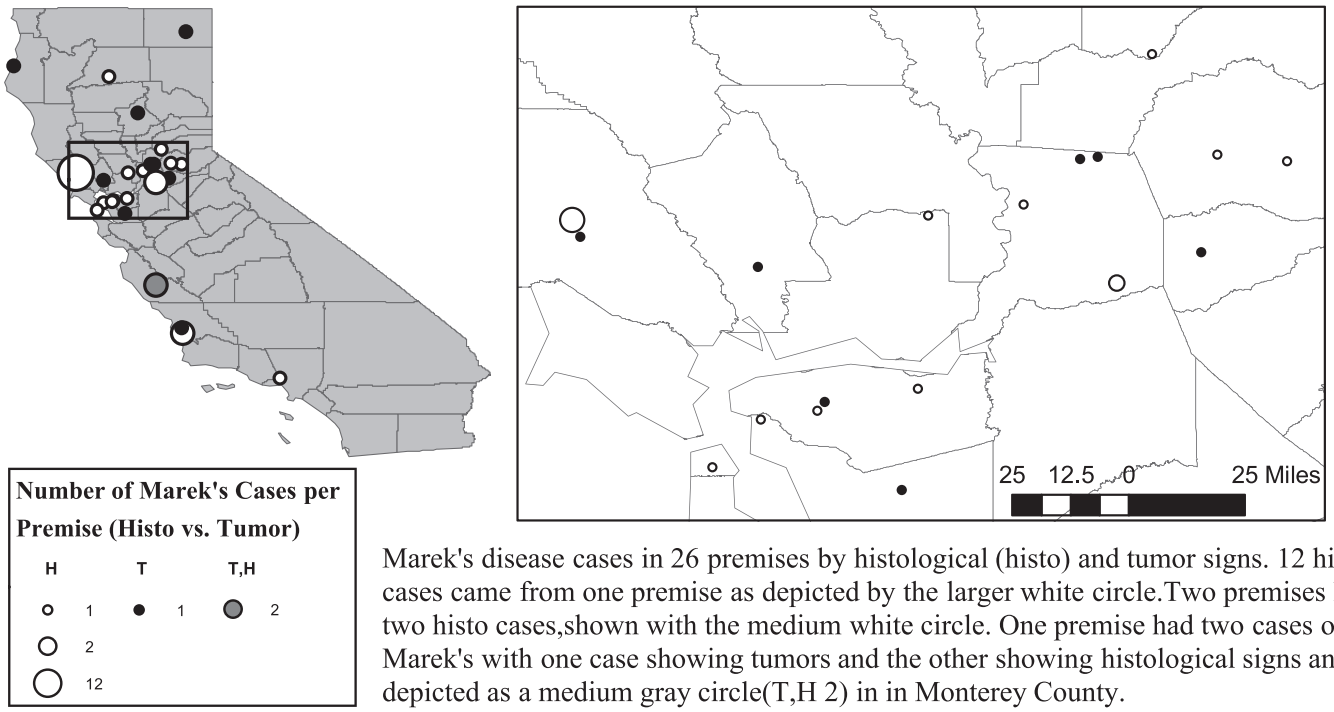

Fig. 2. Distribution of the tumorous (T) *vs.* nontumorous (H) chickens in California.

infiltrations (Fig. 3a). In the brain, up to two to three cell layers of thick lymphocytic perivascular cuffs were included in the (+) group. The infiltrates often had a more varying cellular type as the scores increased from prominent aggregates (++) to confluent sheets (+++) (Figs. 3b,c, respectively). In the heart, one or more tumorous nodules affecting the epicardium or myocardium were given (++) scores. The periportal infiltrations in the liver were scored (++) or more and (+++) only when there was a significantly prominent, expansile lymphoid population with blastoid cells, with minimal or no granulocytes, distinguishing from the small periportal lymphoid aggregates commonly observed in normal chickens. Overall, the (+++) cases were pleomorphic lymphocytes consisting of small, and for the most part, blastoid lymphocytes, sometimes with abnormal large cells and nuclear size, and some cases also contained plasma cells and rare heterophils. Heart and brain were frequently involved in some degree of lymphocytic infiltration ($n = 22$ and $n = 17$, respectively), whereas lung, liver, kidney, and gonads were mostly involved in tumorous birds. The PN lesions were observed in variable locations other than the lumbosacral plexus and the ischiatic nerves, including the eye, mesentery, adrenal gland region, and gastrointestinal wall ganglia. Intranuclear herpes viral inclusions were detected only in the bursal lymphoid neoplasm of Chicken 4, a 6-mo-old Silkie chicken.

From the ocular flock, all but two birds (nos. 33 and 40) had scattered lymphocytic infiltrations in the PNs. Small infiltrates were observed in the brain of five birds (50% of hens with PN lesions), and the hearts had lymphoid infiltrates in all except Chicken 29. A (+) histo score was assigned to the lungs of these birds; however, all birds that had lymphocytic infiltrates had pneumoconiosis, as well.

Vascular lesions were typically observed in moderate to larger arteries, consisting of arteritis and arteriosclerosis comprising mild transmural scattering of small lymphocytes and focal subintimal proliferations, to marked lymphocytic infiltrations with focal aggregations, karyolysis, tunica media hypertrophy, and focal to concentric subintimal proliferations. At least one artery was affected in more than 50% of the cases ($n = 24$), involving the majority of the ocular flock without clinical disease (8/12) and in 15 of the 28 diagnostic cases (Table 2). Of the diagnostic cases with vasculitis, six birds were tumorous, three of which had unknown vaccine status; two birds had not been vaccinated; and one bird was vaccinated. The lesions were multifocal and often involved the arteries surrounding PNs, the major arteries of the heart, or the region around the cranial pole of the kidneys.

Ocular histologic lesions were observed in Chicken 1 among the diagnostic cases and in all 12 hens, in which half had obvious gross ocular changes, ranging from minimal to marked lymphocytic infiltrations in the iris, ciliary body, choroid, the conjunctival subepithelium mostly as perivascular cuffs, and ocular nerves in some birds. The inflammatory infiltrates were more prominent in the chickens that had gross changes (nos. 29–34), composed of pleomorphic lymphocytes with many blast cells, plasma cells, histiocytes, and rare heterophils. Three chickens, including the one diagnostic case, had retinal pigmented epithelial necrosis and separation. Chicken 31, with the unilateral grey iris, had inflammatory plaques and fibrovascular membrane formation in the anterior iris.

Immunohistochemistry. Peripheral nerves were immunostained in eight nontumorous chickens, whereas tumorous peripheral nerves, liver, lung, bursa, thymus, muscle, or skin were included from the 12 tumorous birds. Approximately >90% of the infiltrating cells were positive for CD3 in all cases, with a small fraction of unstained, mixed inflammatory cells that were primarily plasma cells and some large cells or cells with large irregular nuclei. All lymphoproliferative lesions were specifically immunopositive for CD3, confirming the T-cell origin (Figs. 3d–f). In the liver of five tumorous chickens, the staining demonstrated a marked sinusoidal CD3 lymphocyte distribution, in addition to the periportal to parenchymal neoplastic aggregates of cells.

Table 2. Histologic scores of lymphocytic infiltrations in 40 backyard chickens.[A]

Chicken[B]	Brain	PN	Heart	Lung	Liver	Kidney	Gonad[C]	Artery
1	++	++	+	—	+	—	—	
2	++	++	++	+	—	+	—	+
3	+	+	—	—	—	+	—	+
4	++	++	+	+++	++	++	+++	+
5	**NA**	++	**NA**	**NA**	+	+++	**NA**	
6	+	+	+	+	—	+	—	+
7	—	+	+	—	—	—	—	+
8	+	+	—	+++	++	++	++	+
9	—	++	++	—	+	+	—	+
10	++	++	+	—	+	+	—	+
11	—	+	++	—	+	+	—	
12	+	++	++	+++	+++	+++	—	+
13	—	+	—	—	+	—	—	
14	+	+++	+	+++	+++	—	—	
15	++	++	++	+	++	**NA**	**NA**	+
16	+	++	+	+	+	++	— (m)	
17	—	++	++	+++	++	+++	+++ **(m)**	
18	—	+++	++	+++	+++	+++	+++	+
19	+	+	+	—	—	—	—	
20	+	++	—	+	+	—	—	
21	—	+	+	+	+	—	— (m)	+
22	+	+	++	+++	+++	+++	+++	
23	++	+++	+	—	+++	+++	++	+
24	++	+++	+	+	+	++	++	
25	++	+++	+	+	+	—	—	
26	++	+	+	—	+	—	—	+
27	—	+	—	+	+	++	— (m)	+
28	++	++	+	—	++	+	—	+
29	—	+	—	+, P	—	—	—	
30	—	+	+	+, P	—	—	—	+
31	+	+	—	NA	—	—	—	+
32	+	+	+	+, P	—	—	—	+
33	—	—	+	—	—	—	—	+
34	—	+	++	+, P	+	+	—	+
35	+	+	+	+, P	—	—	—	+
36	+	+	+	+, P	+	—	—	+
37	—	+	+	+, P	—	—	—	
38	—	+	+	—	—	—	—	+
39	+	+	+	+, P	—	+	—	
40	—	—	+	+, P	—	—	—	

[A]NA = not available; P = pneumoconiosis.
[B]The 12 chickens with disseminated tumors are shown in boldface.
[C]Gonad represents ovary unless specified male (m).

Immunohistochemistry for CD3 also highlighted the T-lymphocyte population of infiltrating cells in the arterial walls and subintima in sections with vasculitis.

In Chickens 1, 30, and 31, CD3-positive lymphocytes were demonstrated in the iris, ciliary body, and perivascular infiltrates, as well as in the ocular nerves in Chicken 1.

Viral loads; real-time PCR. The viral loads of all MDV serotypes in fresh and fixed spleens of 40 backyard chickens are depicted in Fig. 4. MDV-1 viral loads ranged between 0.01 and 1.3 copies of MDV gB/GAPDH in fixed and between 0.0 and 1.2 copies of MDV gB/GAPDH in fresh spleens of the 12 tumorous chickens. In the 28 nontumorous chickens including the diagnostic cases and the ocular flock hens, MDV-1 values were mostly negative or at very low levels, giving significant differences between tumorous and nontumorous chickens in both fresh ($P = 0.0002$) and fixed ($P < 0.0001$) spleens. Occasional cases were positive on fixed spleen and negative on fresh; however, these were mostly in the nontumorous birds with negligibly small differences, and the results correlated well overall ($R^2 = 0.94$; Fig. 5). The one discrepancy in the tumorous birds was with Chicken 18, which had no MDV-1 viral load in the fresh spleen specimen, although it was 0.014 in the fixed spleen. Chicken 15 from the tumorous group was the only bird with T-cell lymphomas and negative for MDV-1 in fresh and in fixed spleen tissue. MDV-2 virus was detected in fresh and fixed spleens of 2 of the 12 tumorous chickens (nos. 15 and 24), whereas MDV-3 was negative in all tumorous birds. MDV-2 viral loads were encountered at low levels in most of the nontumorous chickens; variably in the fresh or fixed spleens in seven of the diagnostic cases, and in both fixed and fresh spleens in 10 birds from the ocular cases. Three of the seven tumorous birds (nos. 7, 11, and 24) had MDV-2 in both fresh and fixed specimens, whereas no. 24 had the highest load of all studied birds. Chickens 29 and 34 from the ocular flock had no MDV-2 loads in either the fresh or fixed spleens. MDV-3 at low levels were detected in fixed spleens of Chickens 2 and 26 and in fresh spleens of Chickens 3, 7, and 19 in the nontumorous group.

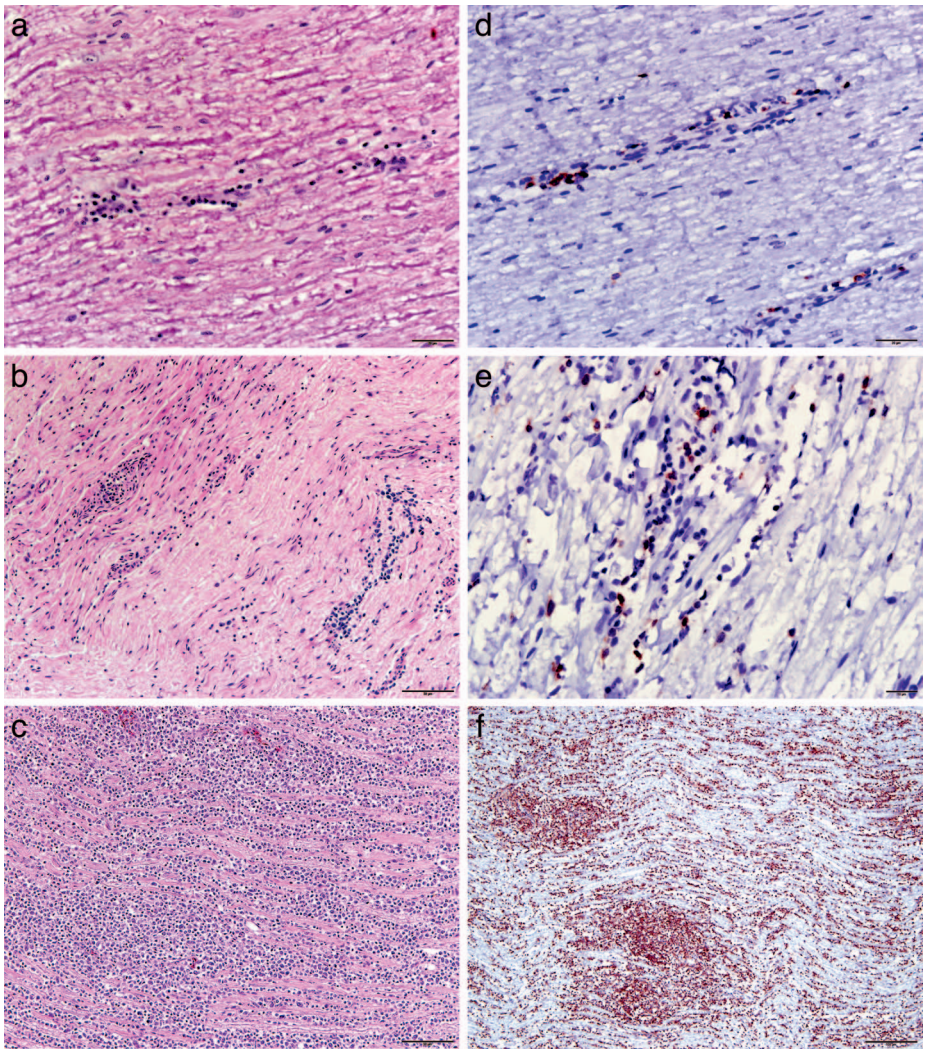

Fig. 3. Histopathologic scoring and CD3 immunohistochemistry of the peripheral nerve infiltrations on H&E sections: nontumorous (H) chickens with (a) few scattered lymphocytes (+); (b) collections of moderate numbers of lymphocytes (++); (c) a tumorous (T) chicken with coalescing to diffuse lymphocytic infiltrations (+++). Immunohistochemical staining of the same cases highlighted the predominantly CD3 population in all histo scores; (d) histo score (+), (e) histo score (++), (f) histo score (+++).

DISCUSSION

This study characterized a population of backyard chickens with spontaneous MD lesions and their spatial distribution in California and developed a real-time qPCR for the three MDV serotypes in frozen and fixed spleens. The study group comprised 28 backyard chickens submitted to the diagnostic laboratory that had clinical disease and lesions compatible with MD, at least as the major differential by histopathology, and 12 chickens in which some had developed ocular MD lesions. The CAHFS laboratories receive a large number of backyard chickens because of the exponential increase in backyard chicken keepers, in addition to the free services provided for up to two backyard chickens (38), of which most succumb to MD (29). The case submissions in this investigation represent a wide geographical distribution throughout the state of California, from the south, Los Angeles region (Tarzana) to the most northern regions (Alturas).

As a general approach, MD is the default diagnosis for lymphoid infiltrations in the nerves of chickens, with reticuloendotheliosis virus (REV) and peripheral neuropathy being the two major differentials for this lesion (43,44). REV is a retrovirus with neurotropic oncogenetic properties similar to MDV-1, and the nonbursal form of REV differentiation from MD is problematic. Nevertheless, REV is not prevalent in the field and was not investigated as a primary or concurrent disease in the current study, except for the one tumorous chicken (no. 15) that was negative for MDV-1, and negative for REV as well, by conventional PCR. Immunohistochemistry demonstrated the T-cell nature of the tumors and the infiltrates in all birds; however, further workup on oncogenes or additional markers to distinguish B-cell lineages or histiocyte populations accompanying the T cells were not conducted, largely because of the lack of readily available chicken markers in routine diagnostic laboratories. Yet, as observed in prior studies (6), nontumorous chickens typically had small, mature lymphoid infiltrations in their nerves, often accompanied by karyorrhexis and plasma cells; a more pleomorphic, blast lymphoid population with mixed inflammatory morphologies was observed in the tumorous chickens. On the other hand, peripheral neuropathy occurs in young birds up to 12 wk of age under experimental conditions (2) and was ruled out in the nontumorous chickens

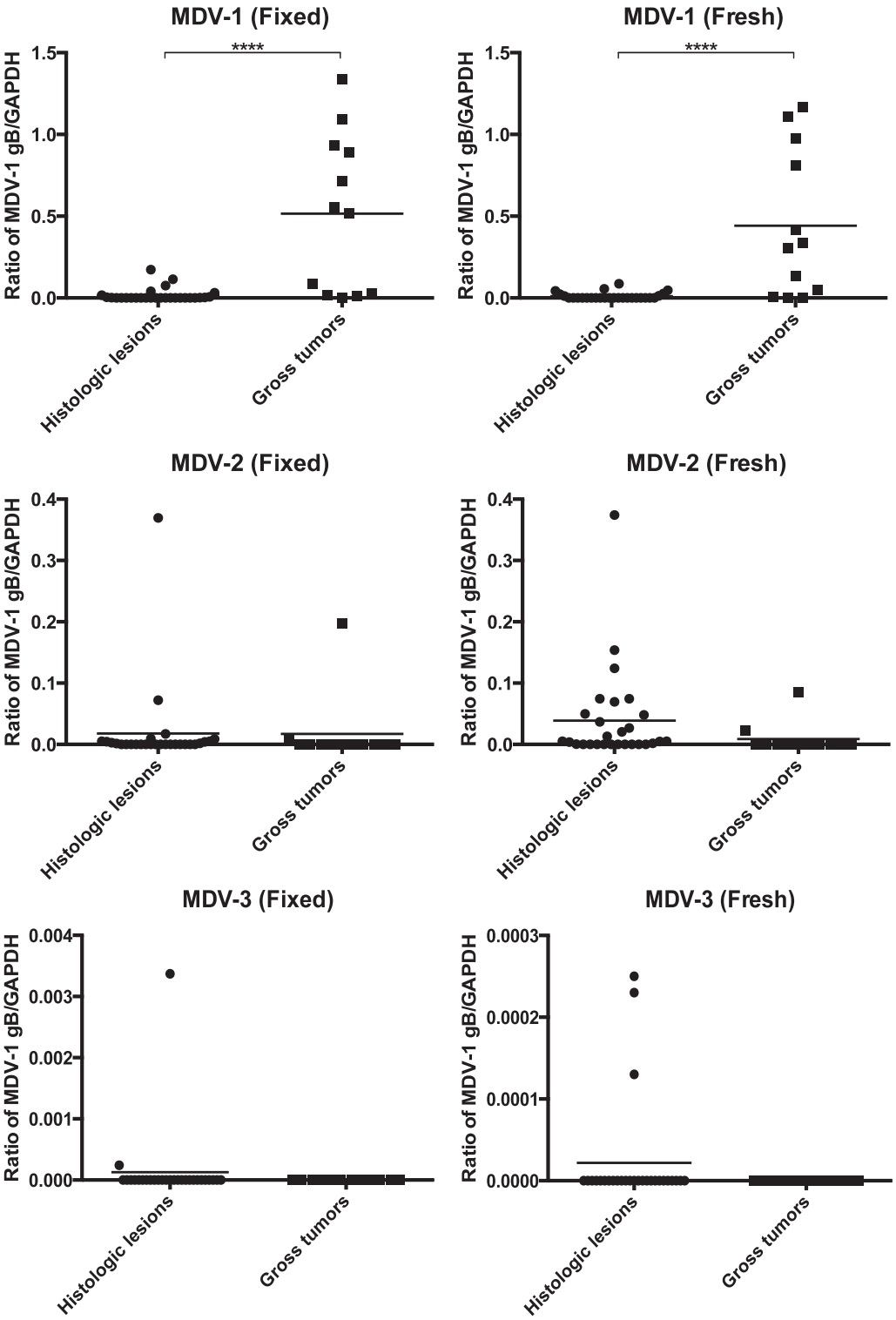

Fig. 4. The real-time qPCR results of MDV-1, MDV-2, and MDV-3 gB/GAPDH DNA ratio in FFPE and fresh spleens from forty backyard chickens with histologic lymphocytic lesions only and gross tumors.

primarily based on the much older ages of the chickens in this study, except for two chickens that were 8 and 12 wk old (nos. 25 and 27, respectively). Of these, one had positive MDV-1 viral loads, and neither demonstrated the type B neural lesions typical of peripheral neuropathy on histology.

Overall, the high histologic lymphocytic infiltration scores corresponded to birds with gross tumors in respective organs, with the exceptions of Chickens 25 and 28. Chicken 25 had a suspicious PN swelling on postmortem examination; however, the case was classified as nontumorous because of an absence of reliable tumor formations similar to Chickens 2, 10, and 21, which correlated well with the low histo scores. In contrast, Chicken 28 had disseminated tumors, but overall low histo scores, because the tumors were not in the tissues included in the histo scores. These differences mark the

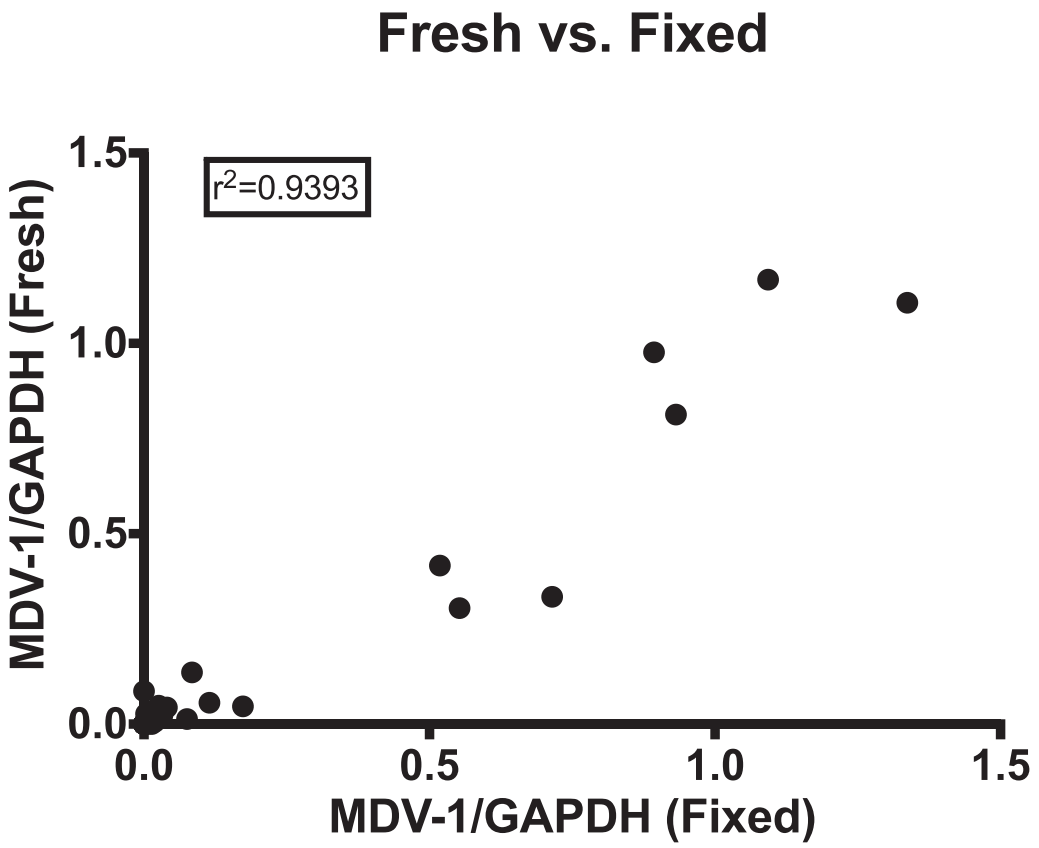

Fig. 5. The correlation analysis of MDV-1 gB/GAPDH in FFPE *vs.* fresh spleens of 40 backyard chickens; $R^2 = 0.94$.

variable nature of lesions in MD, where a segment of a nerve or portion of an organ or organ range will be variably affected grossly, histologically, or both. The assortment of the affected organs in the tumorous birds was as expected (21,43), although skin lesions, which are the most important cause of condemnation in broilers (36), was seen in only two of the birds in our cases.

The age range in the study of backyard chickens complied with late MD in adult chickens previously described in commercial chicken lines (21,42). However, not knowing the MDV pathotype or the maternal antibody or vaccine backgrounds of the backyard chickens, it is not possible to surmise whether these birds had recently been exposed to MDV-1 or had been latently infected with a recent activating trigger, which would be consistent with the "new infection" or "old infection" theories, respectively (42).

The chickens that had only lymphocytic infiltrations in nerves were regarded to have "background" or latent MD. This is regarded as confirmation of exposure to MDV-1 inducing a background inflammatory reaction, potentially with simultaneous neoplastic transformation of T lymphocytes. The true significance of these lesions to the health of the chickens are unknown; however, it is possible that MD-associated immunosuppression may be contributing to disease and mortality since, primarily in the cytolytic phase of MD, chickens may die of secondary infections from immunosuppressive effects (22,36,44). As such, of the nontumorous chickens with histologic MD lesions, 69% had no obvious disease to explain their demise, and immunosuppression may have played a role. Another interesting finding in the present study is the frequency (60%) of arterial changes, with a lesion that has been previously associated with MDV infection (8,32), although the lesions here are more of arteriosclerosis (25) than of atherosclerosis (15). The underlying cause of this observation is not known, and a significant correlation was not observed between the presence of arteritis and arteriosclerosis *vs.* the presence of tumors or the vaccine status of the birds in the known cases; however, the MDV pathotype may be involved.

Ocular MD lesions observed in the one diagnostic case and the 12 chickens from the same flock were all mixed inflammatory infiltrates similar to the experimentally induced lesions, and immunohistochemistry revealed the predominant T-cell population in three of the affected birds (33,37). Similar to the other studies, a distinction between inflammatory and neoplastic cellular infiltrates could not be made, and the one chicken identified with a grey iris had inflammatory changes and anterior synechiae as the underlying cause of the discoloration, which was contrary to our expectation of lymphoid neoplastic infiltrations. The ocular flock also represents the variability of MD and the association of ocular MD with histopathologic nerve lesions rather than with visceral tumors; these 12 hens with similar histories shared the same pen for the entirety of their lives, and only some developed ocular gross and histopathologic lesions compatible with MD, but without apparent neoplastic transformation. Furthermore, all were negative for MDV-1 viral loads, whereas 10 birds (83%) had PN lymphocytic infiltrations. Nevertheless, the development of ocular lesions in vaccinated chickens may still be associated with virulence of the MDV-1 (41).

Vaccination is the primary means of protection from MD and has so far been successful in mitigating losses in the poultry industry. Vaccine strategies, however, remain largely controversial, because the vaccine does not prevent viral transmission and gives nonsterilizing

immunity (20), leading to vaccine failures and evolution of the virus into more virulent strains (35,36). Furthermore, vaccination generally reduces virus shedding (16,30), although this may vary according to the MDV-1 pathotype, the vaccine, and the prior immune status of the bird (39). As also observed in this study, most backyard chickens are either not vaccinated or the vaccine status is unknown (13,40). Many backyard chickens are bred at home, come from neighbors, or are obtained from small feed stores and frequently have unknown virus exposure or vaccination histories. Additionally, the available vaccines for backyard poultry are mostly impractical, and administration problems occur regularly. In the present cases, none of the tumorous birds had MDV-3 (HVT) viral loads, and only two of the nontumorous birds that were known to be vaccinated (nos. 2 and 24) had very low levels, consistent with the observation that backyard chickens are not typically being vaccinated. The MDV-2 virus detected in two (nos. 15 and 24) of the tumorous chickens, both vaccinated, and in most of the nontumorous chickens ($n = 18$) in the fresh, fixed, or both types of spleen that were vaccinated, on the other hand, either represents vaccine strain or, more likely, naturally occurring virus. Three of the tumorous chickens were known to be vaccinated, two of them at 1 day of age. This may represent vaccine break, or a more virulent pathotype of MDV-1 may be at hand, although further speculations cannot be made because the vaccine strain is unknown.

Less stringent vaccination strategies in addition to poor biosecurity in backyard flocks may cause mixing of the MD viral pool in the field, an added risk of emergence of more virulent pathotypes. The vast variation in the genetic line of backyard chickens also may augment this theory or, contrarily, may slow the increase of virulence (20). Additionally, the presence of multiple MDV-1 strains with variable virulence in a single flock or bird providing "passive immunity" to flock mates may be beneficial (5), although competition of the dominant strains may translate into strains with increased virulence (12). Mapping the cases in this study showed the widespread geographic distribution of this population of backyard chickens with MDV-1 viral loads. Given the constant threat of unpredictable MD outbreaks in the poultry industry (36), it is imperative to realize that the close proximity of backyard flocks to commercial operations, at least in California, may call for heightened awareness with regard to "movement" of MD viruses, in addition to other diseases, such as avian influenza.

Development of real-time PCR methods that give absolute or relative quantification of MDV-1 virus and vaccine loads has proven to be useful in aiding in the diagnosis of MD and for evaluating vaccine efficacy (3,18,23). Spleen (fresh) is the primary studied organ (26,45), and FFPE spleen tissue has been shown to be an equally good specimen (7), which was demonstrated in this study as well. Our hypothesis that spleens of tumorous birds will have significantly higher MDV-1 DNA load compared with latently infected chickens was proven for both fresh and FFPE tissues. It is difficult to compare the results of this study with others, because one considerable value of this work is that a highly heterogeneous sample population is used to investigate spontaneous occurrences of MDV in the field with completely unknown pathotypes. It was previously shown that viral loads in tumors are generally 100-fold higher than in latently infected tissues (17). Our results demonstrate up to 10-fold differences in backyard chickens, but only two birds had tumors in the spleen, one having the highest (no. 5) and the other (no. 23) the lowest MDV-1 level in the tumorous group, likely because of sampling the nontumorous regions. Of the nontumorous chickens, nos. 11 and 20 had the highest MDV-1 loads overlapping with the low-end values of the tumorous chickens, and both birds demonstrated generalized clinical signs and had unknown vaccine status and no gross lesions. It remains unknown whether these birds or one of the three chickens that had suspicious nerve thickening on gross exam and high PN histo scores would be more likely to succumb to MD tumors. On the other hand, the MDV-1 loads in these birds may reflect immunization with Rispens, because the primers used in this study cannot distinguish between field and vaccine strains.

Based on the real-time qPCR results, a ratio of MDV-1 gB DNA/GAPDH value of 0.5 and higher in the spleen is highly suggestive of MD tumors. Significant differences in MDV-1 DNA loads between chickens with spontaneous MD tumors and with MD latency in both, fresh frozen, and fixed spleens demonstrate the use of real-time qPCR in the diagnosis of MD. Seeing that splenic MDV-1 loads correlate well with feather pulp viral loads (4,9,10) might instigate a study of the use of feather pulp FTA cards in monitoring MD tumors in live backyard chickens in the future.

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