

Applied Protein and Molecular Techniques for Characterization of B Cell Neoplasms in Horses

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Mature B cell neoplasms cover a spectrum of diseases involving lymphoid tissues (lymphoma) or blood (leukemia), with an overlap between these two presentations. Previous studies describing equine lymphoid neoplasias have not included analyses of clonality using molecular techniques. The objective of this study was to use molecular techniques to advance the classification of B cell lymphoproliferative diseases in five adult equine patients with a rare condition of monoclonal gammopathy, B cell leukemia, and concurrent lymphadenopathy (lymphoma/leukemia). The B cell neoplasms were phenotypically characterized by gene and cell surface molecule expression, secreted immunoglobulin (Ig) isotype concentrations, Ig heavy-chain variable (IGHV) region domain sequencing, and spectratyping. All five patients had hyperglobulinemia due to IgG1 or IgG4/7 monoclonal gammopathy. Peripheral blood leukocyte immunophenotyping revealed high proportions of IgG1- or IgG4/7-positive cells and relative T cell lymphopenia. Most leukemic cells lacked the surface B cell markers CD19 and CD21. *IGHG1* or *IGHG4/7* gene expression was consistent with surface protein expression, and secreted isotype and Ig spectratyping revealed one dominant monoclonal peak. The mRNA expression of the B cell-associated developmental genes *EBF1*, *PAX5*, and *CD19* was high compared to that of the plasma cell-associated marker *CD38*. Sequence analysis of the IGHV domain of leukemic cells revealed mutated Igs. In conclusion, the protein and molecular techniques used in this study identified neoplastic cells compatible with a developmental transition between B cell and plasma cell stages, and they can be used for the classification of equine B cell lymphoproliferative disease.

Lymphoproliferative disorders include a spectrum of neoplasms, ranging from those that manifest with primary extramedullary tissue involvement (lymphoma) to those with primary blood or bone marrow involvement (leukemia). Lymphoid neoplasms can be further characterized by phenotype (B or T cell) and stage of maturity (precursor or mature cell). Some patients present with concurrent lymphoid tissue tumors and leukemia, complicating classification as lymphoma or leukemia, and these are frequently grouped as precursor lymphoma/leukemia or mature lymphoma/leukemia (1). In the case of mature B cell neoplasms (i.e., chronic lymphocytic leukemia [CLL] or small lymphocytic lymphoma [SLL]), the two neoplasms are distinguished by the main site of involvement. In CLL, most tumor cells are located in the blood (>5,000 cells/ μ l), although some may infiltrate and develop masses in lymphoid tissues (1). In contrast, SLL presents with lymphadenopathy due to neoplastic small lymphocytes but lacks the peripheral blood lymphocytosis characteristic of CLL (2, 3). In human patients, mature B cell neoplasms express cell surface markers distinct from those of normal B cells and either somatically mutated or unmutated immunoglobulin heavy-chain variable (IGHV) genes (4). The mutational status of IGHV genes has been used as a prognostic indicator of B cell CLL (B-CLL) and for speculation of the developmental origin of neoplastic cells in human patients (5).

B cell neoplasms that arise from differentiated mature cells may have undergone somatic hypermutation and productive Ig heavy-chain class switching in germinal centers (6). These cells may produce excessive monoclonal Ig detectable in serum and/or urine (7). The most common form of lymphoid neoplasia in horses is T cell-rich large B cell lymphoma, which is composed of large neoplastic B cells, with an accompanying infiltrate of non-

neoplastic small mature T cells (8, 9). Other variants of neoplasms diagnosed in horses include peripheral T zone lymphoma, diffuse large B cell lymphoma, and lymphoma of granular lymphocytes (8). Acute or chronic lymphoid leukemias are reported less frequently in horses than in dogs and cats (10–13). In a recent case series of lymphoma in 203 horses, only 2% presented with leukemia (8). There have been some reports of concurrent B cell lymphoma and leukemia in horses; however, expanded cell marker expression and IGHV sequencing were not pursued in those studies (3, 9–11, 14–16). Horses have an IGHV domain repertoire as diverse as that in humans and sheep, with a similar profile of gene usage during developmental progression (17, 18).

The classification of lymphoproliferative disorders is essential for the diagnosis and improvement of therapeutic regimens, both of which are in great need in equine medicine. The World Health Organization (WHO) classification is based on a combination of

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clinical data, course of disease, cell morphology, immunophenotyping (immunocytochemistry or histochemistry and flow cytometry), and genetic features (1). The WHO system has been adapted for the classification of canine and equine lymphomas based on cell morphology and immunohistochemistry for a few immunologic markers (8, 19).

The aim of this study was to use the expression of genes and cell surface molecules, secreted Ig isotypes, IGHV domain sequencing, and Ig spectratyping to characterize a rare clinical condition of mature B cell neoplasms characterized by leukemia and lymphadenopathy (mature lymphoma/leukemia) in 5 equine patients. This study offers an advancement in the diagnosis and classification of equine lymphoid neoplasms, with potential applications in future studies that evaluate treatment protocols in this species.

MATERIALS AND METHODS

Experimental design. The experimental studies were approved by the Cornell University Center for Animal Resources and Education and Institutional Animal Care and Use Committee for the use of vertebrates in research. This study performed assays using blood samples from 5 equine patients diagnosed with mature B cell leukemia, hyperglobulinemia, monoclonal gammopathy, and concomitant lymphadenopathy (Table 1) submitted to the Equine Immunology Laboratory at the Cornell University College of Veterinary Medicine for lymphocyte immunophenotyping and serum Ig isotype quantification between 2000 and 2009. Clinical cases were managed in their respective referral teaching hospitals (horse 1 at the Ohio State University Veterinary Medical Center, horses 2, 3, and 5 at the Cornell University College of Veterinary Medicine, and horse 4 at the University of Pennsylvania School of Veterinary Medicine). Clinical examination, history, clinical pathological, and postmortem findings (when available) were provided by the respective institutions; complete clinical records were not available for horse 1. Blood samples from ≥ 1 healthy research adult horse (of various breeds) were tested side by side with each patient sample as assay controls for immunophenotyping using flow cytometry. RNA archived from blood samples from 3 healthy research mares (2 Thoroughbred and 1 pony; age range, 10 to 15 years) and from bone marrow and mesenteric lymph node samples from 1 healthy adult Thoroughbred research gelding (unknown age) were processed side by side with leukemic samples as assay controls in the standard and quantitative reverse transcriptase PCR (RT-PCR) assays.

Protein electrophoresis and immunoglobulin isotype concentrations. Agarose gel protein electrophoresis analyses with respective electrophoretograms were performed using serum or plasma samples at the Cornell University Clinical Pathology Laboratory, which provided an in-house established reference interval for serum but not plasma electrophoresis. Plasma was the only available sample type from horse 3 for protein electrophoresis. Serum IgM, IgG, and IgA concentrations were determined using commercially available radial immunodiffusion (RID) kits for horses, as per the manufacturer's instructions (VMRD, Pullman, WA). A standard curve was generated with the known concentrations of purified equine Igs provided in the kit and their respective precipitate diameters. The concentrations of IgM, IgG, and IgA in each serum sample were determined by comparing the individual precipitate diameters to those of the standard curve. Serum samples were diluted 2-fold when values were greater than the upper limit of the standard curve. Reference intervals for serum Igs were published previously (20). In addition, serum (or plasma from horse 3) IgG concentrations were measured using an immunoturbidimetric assay on an automated chemistry analyzer at the Cornell University Clinical Pathology Laboratory (Midland Bioproducts Corporation, Boone, IA, and Hitachi P-Modular; Roche Diagnostics, Indianapolis, IN).

Horses have 11 immunoglobulin heavy-chain constant genes in the IGH locus named and ordered as *IGHM*, *IGHD*, *IGHG1*, *IGHG2*, *IGHG3*, *IGHG7*, *IGHG4*, *IGHG6*, *IGHG5*, *IGHE*, and *IGHA* (20, 21). The *IGHG7*

TABLE 1 Clinical data of affected horses

Affected horse/ reference interval	Signalment	Clinical signs ^a	Count ($\times 10^3/\mu\text{l}$) of:					Total protein (g/dl)
			HCT (%) ^b	Hb (g/dl) ^c	Platelets	Neutrophils (no. of bands)	Lymphocytes	
1	17-yr-old Haflinger mare	Lymphadenopathy, respiratory disease	NA	NA	NA	NA	37.0	10.8
2	7-yr-old Thoroughbred gelding	Wt loss, dermatitis, multiple nodules palpable in abdomen (rectal examination)	41	15.6	79.0	4.1 (0)	5.3	12.1
3	14-yr-old Clydesdale mare	Mandibular, prescapular, cervical lymphadenopathy, wt loss, lethargy, fever, ventral edema, tachycardia, diarrhea, bilateral epistaxis, inspiratory stridor, pleural fluid	24	9.3	16.0	2.2 (2.2)	103.8	9.0
4	28-yr-old Quarter Horse gelding	Mandibular lymphadenopathy, fever, ventral edema, respiratory stridor and distress, tachycardia, PPID	37	13.8	NA (adequate on smear)	6.9 (0.8)	68.0	8.8
5	11-yr-old Clydesdale mare	Bilateral eyelid edema, limb edema, decreased appetite, lethargy, chronic wt loss, intraperitoneal mass (US)	29	10.5	151.0	6.2 (0)	17.0	10.9
Reference interval			34–46	11.8–15.9	94.0–232.0	2.7–6.6	1.2–4.9	5.7–7.7

^a PPID, pituitary pars intermedia dysfunction; US, ultrasound exam.

^b HCT, hematocrit; NA, not available.

^c Hb, hemoglobin.

TABLE 2 RT-PCR and qRT-PCR SYBR primer sets

Gene by primer type	GenBank accession no.	Forward (5'-3') sequence	Reverse (5'-3') sequence	Product size (bp)	Annealing temp (°C)	Primer concn (nM)
RT-PCR primers						
CD34	XM_001491596	CTAGGGTGTGCTCCTTGCTC	GACCAAGTCAATCAGGGTCT	209	58	500
RAG2	AF447533	CACCAAACAATGAGCTTTTCG	TTTGGGTGGAAGGGATGTAG	207	58	500
CD11b	XM_001495590	GGGCAGCCCTGACAGTA	GCTGATGCCCAGTCCTGA	131	58	500
CD38	XM_001498785	ATGGCCAACCACAGATTTCAG	CCAGATGTGCAAGATGGATG	923	58	500
ELA-DRA	JQ254080	CTCCAGAGGTGACTGTGCTC	TGTCTCTGAGAGGGGAGTTG	311	58	500
qRT-PCR primers						
CD38	XM_001498785	GGCAGATGCTACACCTACA	TATGAGCGGTTGATAGTCTTGT	141	60	500
IGHG1	XM_001496465	CCAGCGAGACCTACATCT	TGGTGGTTGTTGGGAATG	88	60	500
IGHG4/7	ECA302057 , ECA302058	GCTCACTGTGGAGACTAA	GAGACTTGGAGACGGATT	107	57	500
IGKC	KJ741386	TTCAGTGGCAGTGGATCTGG	CAGAAGACGGTGGGAAGATG	184	63	500
IGLC	KF985132	TCCAGGCTGAGGACGAGGC	GGAAGAGAGAGACCGAGGGT	135	63	300

gene has a high homology to the equine IGHG4 gene and the IGHG3 to IGHG5 genes; the description of the equine immunoglobulin heavy-chain genes substituted the formerly designated IgGa (now IgG1), IgGb (IgG4/7), IgGc (IgG6), and IgGT (IgG3/5) (20, 22). Serum IgG1 (IgGa, clone CVS48; AbD Serotec, Raleigh, NC) and IgG4/7 (IgGb, clone CVS39; AbD Serotec) isotype concentrations were determined at the Cornell University Equine Immunology Laboratory using an enzyme-linked immunosorbent assay (ELISA) (23, 24). Briefly, goat anti-horse IgG(H+L) was coated onto ELISA plates as a capture antibody for the serum Igs (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Serial dilutions (1:10² to 1:10⁸) of serum samples and horse Ig isotype reference serum were tested in triplicate (Bethyl Laboratories, Inc., Montgomery, TX). Murine monoclonal antibodies against equine IgG1 (IgGa) and IgG4/7 (IgGb) were used as the detection antibody. Peroxidase-conjugated goat anti-mouse IgG(H+L) antibodies detected bound mouse monoclonal antibodies (Jackson ImmunoResearch Laboratories, Inc.). A standard curve was generated from the known reference serum dilutions and their respective optical density (OD) values (Thermo Fisher Scientific, Waltham, MA). The concentrations of serum Ig isotypes in each testing sample were determined from the standard curve. The reference intervals were published previously (23, 24).

Peripheral blood leukocyte immunophenotyping. Peripheral blood leukocyte immunophenotyping was performed using monoclonal antibodies and flow cytometric analysis at the Cornell University Equine Immunology Laboratory (23). Briefly, peripheral blood mononuclear cells (PBMC) (10⁶) were isolated from heparinized blood using Ficoll density centrifugation. The cell surface molecules tested with monoclonal antibodies included CD2 (clone HB88a), CD4 (clone HB61A), CD5 (clone HT23A), and CD8 (clone HT14A) from the Washington State University Monoclonal Antibody Center, Pullman, WA; CD3 (F6G.3G12) from M. Blanchard, University of California—Davis, CA; CD19-like (CZ2.1), major histocompatibility complex (MHC) class I and class II (CZ3 and CZ11, respectively), and lymphocyte function-associated antigen (LFA-1 or CD11a/CD18, CZ3.2) from D. Antczak, Cornell University, Ithaca, NY; CD21 (B-ly4) from BD Biosciences, San Jose, CA; IgM (CM7), IgA (K1292G5), IgGa (CVS48), and IgGb (CVS39) from AbD Serotec, Raleigh, NC; IgGc (CVS53) and IgGT (CVS40) from P. Lunn, North Carolina State University, Raleigh, NC; and an irrelevant molecule (negative-control against canine parvovirus, C. Parrish, Cornell University) (25–27). The secondary-stage antibody was a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG(H+L) antibody (Jackson ImmunoResearch Laboratories, Inc.). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Leukocyte subpopulations (lymphocytes, monocytes, and neutrophils) were identified and gated based on their characteristic size and complexity in a forward-scatter (FSC) and side-

scatter (SSC) dot plot. The percentage of cells in the lymphocyte gate positive for each marker was measured using histogram plots of fluorescence intensity (i.e., stronger fluorescence than the irrelevant control). The reference intervals were published previously (23, 24).

Reverse transcriptase PCR. The expression of 31 genes associated with lymphoid origin and early or late stages of B cell development, including plasma cells, was qualitatively assayed by standard RT-PCR. Total RNA was isolated from leukocytes and assay control tissue samples using the RNeasy minikit with on-column digestion for genomic DNA, according to the manufacturer's instructions, as described previously (Qiagen, Inc., Valencia, CA) (27). The relative purity and quantity of the isolated RNA were determined by spectrophotometry using NanoDrop 1000 (Thermo Fisher Scientific). The RevertAid reverse transcriptase kit was used to synthesize oligo(dT)-primed cDNA (Thermo Fisher Scientific). The amplification reaction mixtures contained 1× DreamTaq buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM forward and reverse primers specific for each gene, 1.25 U DreamTaq DNA polymerase (Thermo Fisher Scientific), and 25 ng of cDNA template. The primers used are listed in Table 2 or were previously published (23, 28, 29). The thermal cycling parameters were 95°C for 5 min; 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 30 s; and a final extension of 72°C for 10 min. Amplification products were run on 1% agarose gels and stained with GelGreen nucleic acid stain (Phenix Research Products, Candler, NC) for visualization with the Gel Doc EZ system (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time RT-PCR. Quantitative RT-PCR (qRT-PCR) was used to measure the expression of nine essential genes for B cell development in RNA isolated from leukocytes and control tissue samples (28). Reactions were performed in triplicate with 10 ng of RNA (leukocyte, bone marrow, or lymph node), primers (Table 2), and the iScript one-step RT-PCR kit with SYBR green mix in a CFX96 real-time PCR detection system (Bio-Rad Laboratories). In addition, a no-template control was included on each plate in triplicate. The cycling parameters were 1 cycle of 50°C for 10 min, 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s, and then annealing for 30 s, followed by melt curve analysis (see Table 2 for annealing temperatures). The amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run. SYBR primers for *ACTB*, *CD19*, *EBF1*, *IGHM*, and *PAX5* were validated previously (28). For the present study, SYBR primers spanning intron/exon boundaries for *CD38*, *IGHG1*, *IGHG4/7*, *IGKC*, and *IGLC* were designed with Beacon Designer 7.91 software (PREMIER Biosoft International, Palo Alto, CA) and validated on RNA standard curves. RNA standard curves were prepared for each gene by *in vitro* transcription with the TranscriptAid T7 high-yield transcription kit (Thermo Fisher Scientific) from linearized clones harboring the respective gene. *In*

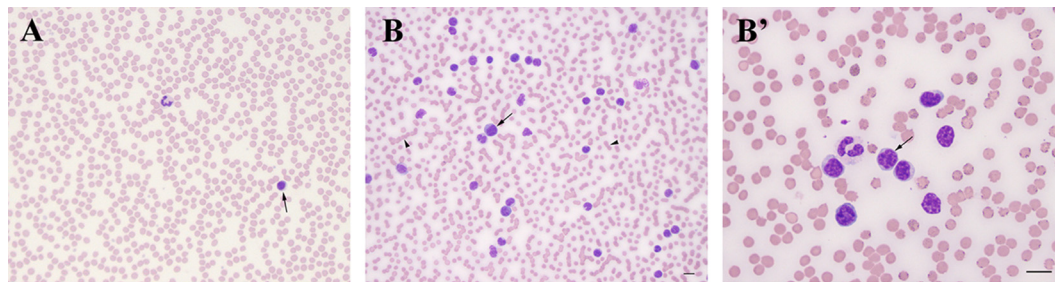


FIG 1 Representative photomicrograph of venous blood from a clinically healthy horse and a horse with leukemia. (A) In the healthy horse, a neutrophil and small lymphocyte (8 to 10 μm , arrow) are present, and erythrocytes are uniformly dispersed with no rouleaux formation. (B [low power]) In horse 3 with leukemia, there is a marked lymphocytosis, consisting of small cells (8 to 10 μm) with a few large lymphocytes (arrow). The small lymphocytes have clumped chromatin and small amounts of light blue cytoplasm. Some have lobulated or irregular nuclei. There is a blue hue to the background, and excessive rouleaux formation is evident in erythrocytes (arrowheads), indicating increased plasma protein and globulins, respectively. (B' [high power]) The small size of the lymphocytes is apparent in relation to the toxic segmented neutrophil. One of the lymphocytes has a lobulated nucleus (arrow). Scale = 10 μm , Wright's stain.

vitro-transcribed RNA was purified with RNA Clean & Concentrator columns (Zymo Research, Irvine, CA) and quantified with a Nano-Drop 1000 (Thermo Fisher Scientific). Ten-fold serial dilutions were made for the standard curve. The reaction efficiency ranged between 92.8 and 102.8% (the slope of the curve ranged between -3.256 and -3.506), and no primer dimers were observed on the melt curve analysis. Absolute quantification of mRNA transcript numbers was determined from the RNA standard curve with the CFX Manager software (Bio-Rad Laboratories).

Amplification of the equine IGHV domain. Immunoglobulin heavy-chain variable region sequences were amplified from leukocyte cDNA of all leukemic samples, with conserved primers spanning 89% of the VDJ region (5'-GTGGTTCTTCTCTTTCTGGTG-3' and 5'-CCTGAGGAGACGGTGACCAAG-3') and a proofreading polymerase (Bio-Rad Laboratories) (18). PCR products were visualized on a 1% agarose gel, directly purified using the GeneJET PCR purification kit, and ligated into CloneJET PCR cloning kit (Thermo Fisher Scientific). 5-Alpha competent *Escherichia coli* strains were transformed with ligation products, and single colonies were grown in liquid broth with ampicillin (New England BioLabs, Inc., Ipswich, MA). Plasmid DNA was isolated from ≥ 10 clones per sample with the GeneJET plasmid miniprep kit (Thermo Fisher Scientific) and sequenced at the Cornell University Institute of Biotechnology Genomics Facility. Sequences were analyzed with the Geneious 6.1.5 software (Biomatters Ltd., Auckland, New Zealand). Percent nucleotide identity to the germ line IGHV gene was determined by comparing the leukemic IGHV sequence against the equine reference genome EquCab2.0 with the NCBI BLAST tool (18).

Immunoglobulin spectratyping. Fluorescent Ig heavy-chain variable region amplicons were obtained as described above in conjunction with the M13 tail fluorescent labeling method (30). The amplification reaction mixtures were composed of leukocyte cDNA, $1\times$ DreamTaq buffer, 0.2 mM dNTPs, 1.25 U of DreamTaq DNA polymerase, 1 μM M13 forward primer labeled with 6-carboxyfluorescein (FAM) (5'-FAM-TGTAACACGACGGCCAGT-3'), 0.07 μM conserved Ig forward primer with an M13 tail (5'-TGTAACACGACGGCCAGTGTGGTCTTCTCTTCTGGT G-3'; M13 tail underlined), and 1 μM conserved Ig reverse primer (5'-CTGAGGAGACGGTGACCAAG-3') (Thermo Fisher Scientific). The primers were commercially synthesized (Eurofins MWG Operon, Huntsville, AL). Touchdown thermal cycling was performed with an initial 5 min of denaturation at 95°C, 4 cycles in which the annealing temperature decreased 2 degrees each cycle from 68°C to 60°C (95°C for 30 s, annealing for 30 s, and 72°C for 1 min), which continued for 36 cycles at 60°C, followed by a final extension at 72°C for 10 min. PCR products were visualized on a 1% agarose gel with GelGreen nucleic acid stain before fragment analysis submission (Phenix Research Products, Candler, NC). To obtain the spectratype, 1 μl of PCR product was premixed with 15 μl of Hi-Di formamide and 0.5 μl of GeneScan 500

LIZ dye size standard (Life Technologies, Grand Island, NY). Samples were denatured at 95°C for 5 min. Fragment analysis was performed on the ABI 3730xl DNA analyzer at the Cornell University Institute of Biotechnology Genomics Facility, Ithaca, NY. Electropherograms were analyzed with the Applied Biosystems Peak Scanner software version 1.0 (Life Technologies).

Statistical analysis. A Shapiro-Wilk normality test using the GraphPad software (GraphPad, San Diego, CA) revealed that the gene expression data (quantitative RT-PCR for *EBF1*, *PAX5*, *CD19*, *IGHM*, *CD38*, *IGKC*, and *IGLC*) was not normally distributed, and the nonparametric Mann-Whitney-Wilcoxon rank sum test was performed for two-way comparisons between the leukemic ($n = 5$) and control healthy ($n = 3$) horse samples. The alpha value was 0.05. Other data were presented descriptively.

Nucleotide sequence accession numbers. The equine IGHV sequences determined in this study are available in GenBank with the accession numbers KJ741369 to KJ741385. Other accession numbers for the primers used are listed in Table 2.

RESULTS

The aim of this study was to use the expression of genes and cell surface molecules, secreted Ig isotypes, IGHV domain sequencing, and Ig spectratyping to characterize mature B lymphoma/leukemia in 5 equine patients. The application of molecular techniques to equine lymphoid neoplasms advances the classification of neoplastic cells, in anticipation of better informing diagnosis and gaining insight into the underlying mechanisms.

Clinical and histopathological findings. The 5 horses with concurrent mature B cell lymphoma/leukemia were of both sexes and different breeds, with an age range of 7 to 28 years old (Table 1). Three horses presented with peripheral lymphadenopathy, whereas intracavitary masses were detected by rectal examination or transabdominal ultrasound examination in the remaining two horses. Two of 4 horses with complete hemogram results had mild anemia, and 2 were also thrombocytopenic. Only 1 horse was neutropenic, and this horse also had a left shift and toxic changes evident in neutrophils on blood smear examination. All horses had lymphocytosis, but this was mild in 1 horse. Circulating tumor cells were a mixed population of small and intermediate cells (8 to 12 μm) with indented nuclei, containing clumped chromatin, no nucleoli, and a small amount of light blue cytoplasm. Low numbers of large lymphocytes (15 to 20 μm) with fine chromatin and indistinct nucleoli were seen in 3 horses, comprising $<6\%$ of the total lymphocytes (Fig. 1). In two horses, some of the tumor

TABLE 3 Cytologic and/or histopathologic findings

Affected horse	Blood smear cytology	Bone marrow cytology	Lymph node cytology	Gross pathology	Histopathology
1	Not available	Not performed	Not performed	Not performed	Not performed
2	Lymphocytes with excess cytoplasm and cleaved mature nuclei	Not performed	Not performed	Multifocal firm nodules on intestinal serosa; diffusely enlarged mesenteric lymph nodes	Various sizes of proliferating lymphoblasts in mesenteric lymph nodes, intestinal submucosa, kidneys (perivascular), heart, and liver (periportal); multicentric lymphoblastic lymphoma
3	99% small to intermediate lymphocytes with 1% blasts; bilobed, lobulated, indented, or cleaved nuclei	Not performed	Not performed	Diffuse lymphadenopathic tumor nodules in lungs, heart, liver, kidneys, spleen, and GI tract; diffuse petechiations; mandibular, prescapular, superficial cervical, and mesenteric lymphadenopathy ^a	Pleomorphic neoplastic cells (7–15 μ m); scant eosinophilic cytoplasm; centrally to peripherally located nucleus; coarse chromatin with multiple nucleoli; moderate population of plasma cells present diffusely in the lamina propria; CD79a- and BLA36-positive cells; multicentric T cell-rich large B cell lymphoma ^b
4	68% leukocytosis due to 12- to 15- μ m atypical lymphocytes; round to indented nucleus; granular to stippled chromatin; indistinct nucleoli; scant dark cytoplasm	Hemodiluted sample not diagnostic	Small mature lymphocytes with moderate no. of medium-sized lymphocytes and few lymphoblasts; no evidence of neoplasia	Diffuse lymphadenopathy; tumor nodules in lungs, liver, kidneys, and intestines	Neoplastic lymphocytes present in abdominal parietal subperitoneal mass; aortic wall, lymph nodes, pituitary gland, lungs, liver, kidneys, intestines, and lymph nodes slightly increased; neoplastic cells in vessel multicentric lymphosarcoma
5	Intermediate to large mononuclear cells; deeply indented to cleaved nuclei; small amt of light blue cytoplasm	Not performed	Not performed	Not performed	Not performed

^a GI, gastrointestinal.^b CD, cluster of differentiation; BLA36, B lymphocyte antigen 36.

TABLE 4 Serum protein electrophoresis results (g/dl)

Affected horse/ reference interval	Albumin	α 1-Globulin	α 2-Globulin	β 1-Globulin	β 2-Globulin	γ -Globulin	Total
1	1.64	0.51	1.12	0.82	6.52	0.50	11.10
2	2.96	0.73	1.41	1.11	0.47	6.93	13.60
3 ^a	2.13	0.26	1.11	0.88	0.99	3.13	8.50
4	2.44	0.35	0.99	0.90	0.68	2.13	7.50
5	2.10	0.41	0.91	0.93	0.45	6.10	10.90
Reference interval	2.3–3.5	0.3–0.8	0.7–1.3	0.2–1.1	0.3–0.8	0.7–1.8	5.7–7.9

^a Electrophoresis was performed on heparinized plasma from this horse; reference intervals for plasma were not determined.

cells had abnormal morphological features, including clover-leaf-shaped, flower-like, and monocytoid nuclei. Excessive rouleaux formation was evident in the erythrocytes in 3 horses on blood smear examination (Fig. 1).

Biochemical profiles were determined in 3 horses. Horse 3 had abnormalities attributable to concurrent liver injury (aspartate aminotransferase [AST] level, 534 U/liter; reference interval, 212 to 426 U/liter; alkaline phosphatase [ALP] level, 412 U/liter; reference interval 75 to 220 U/liter) and diarrhea (decreased electrolytes and high anion gap titration metabolic acidosis). When horses 3 to 5 were tested, the results for creatinine and total calcium levels were within reference intervals (creatinine, 1.5 to 1.8 mg/dl; reference interval, 0.9 to 1.8 mg/dl; total calcium, 11.3 to 12.0 mg/dl; reference interval, 11.2 to 13.0 mg/dl). Mild hyperphosphatemia was present in horses 3 and 5 (4.9 and 5.9 mg/dl, respectively; reference interval, 2.1 to 4.7 mg/dl). Bone marrow aspirates from horse 4 were uninformative due to poor sample quality from hemodilution; in the same horse, lymph node biopsy imprint smear cytology resulted in no evidence of neoplasia but reactive lymphoid hyperplasia.

Necropsy was performed in 3 horses (Table 3). Gross pathological examination revealed diffuse lymphadenopathy involving mesenteric (horses 2 and 3) or cervical and mediastinal (horse 4) lymph nodes and variably sized tumor nodules in multiple internal organs, including the gastrointestinal tract (all 3 horses) and thoracic organs (horses 3 and 4). Histopathologic examination of tissue samples collected during necropsy revealed neoplastic lymphocytes in the lymph nodes of all 3 horses and perivascular infiltrates in the intestinal tract, kidney, heart, and liver. In addition, multifocal to coalescing or nodular infiltrates of neoplastic lymphocytes were found in various tissues of all 3 horses, including the intestine, heart, liver, lungs, spleen, pancreas, mammary gland, aortic and abdominal wall, and pituitary gland. Bone marrow involvement was evident as a single lobulated mass in the proximal femur of horse 3. Immunohistochemical staining for CD3, CD79a, and B lymphocyte antigen 36 (BLA36) was performed on the large intestine and bone marrow of horse 3, and the results showed a mixed population of lymphocytes, with large B cells and small T cells, consistent with a diagnosis of a T cell-rich B cell lymphoma.

Protein electrophoresis. Protein electrophoresis revealed hyperglobulinemia (range, 7.50 to 13.60 g/dl) in all cases and hypoalbuminemia in 3 horses (Table 4). The hyperglobulinemia was attributed to a monoclonal peak in the gamma region in 4 cases (gammaglobulin range, 2.13 to 6.93 g/dl) and 1 case in the beta 2-globulin region (horse 1, 6.52 g/dl) (Fig. 2). Horse 3 also presented with an increased beta 2-globulin (small peak) value attributed to fibrinogen (plasma sample).

Serum immunoglobulin isotype concentration. Serum Ig

isotype-specific quantitation using radial immunodiffusion revealed IgG hyperglobulinemia in ≥ 2 horses (Table 5). However, when using the immunoturbidimetric assay, all horses had markedly increased serum IgG concentrations. The serum IgM concentration was low (range, ≤ 25 to 70 mg/dl) in all affected horses. When tested, the serum IgA concentration was normal and low in 2 horses and 1 horse, respectively. The IgG serotype ELISA revealed high levels of IgG1 (IgGa; range, 2,862 to 11,025 mg/dl) and IgG4/7 (IgGb; 5,835 and 7,817 mg/dl) in 3 and 2 horses, respectively. Serum IgG3/5 was also elevated for horses 3 and 4 (IgGT; 687 mg/dl and 862 mg/dl, respectively).

Peripheral blood leukocyte immunophenotyping. Peripheral blood leukocyte immunophenotyping revealed markedly increased percentages of leukemic IgG-positive cells and concomitant T cell lymphopenia (CD3 range, 1.1 to 11.0% positive cells; Table 6) in all horses. The majority of leukemic cells did not express the B cell marker CD19 (range, 0.2 to 14.1%) or CD21 (range, 1.5 to 8.6%) (Fig. 3). For horse 3, the percentage of IgM-positive, CD19⁺, or CD21⁺ cells suggested the presence of a residual population of normal B cells. Three horses had a high percentage of IgG1 (IgGa; range, 75.9 to 98.1%)-positive cells, whereas 2 horses had a high percentage of IgG4/7 (IgGb; 87.7 and 93.4%) cells. The surface IgG expression matched the secreted IgG isotype on ELISA (Tables 5 and 6). In all cases, almost all cells in the lymphocyte-gated area were positive for MHC classes I (range, 99.9 to 100%) and II (range, 96.4 to 99.5%) and the integrin CD11a/CD18 or LFA-1 (range, 98.3 to 100%).

Gene expression. The isolated peripheral blood leukocytes from all horses expressed all leukocyte signature genes using the qualitative RT-PCR test, albeit with some variability of band intensity (see Fig. S1 in the supplemental material). Therefore, the expression of selected genes was measured using quantitative analysis. The mRNA copy numbers for *EBF1*, *PAX5*, and *CD19* in affected horses were greater ($P = 0.02$) than those in the control healthy horses but were similar to those of the control horses for *IGHM* ($P = 0.3$) and *CD38* ($P = 0.07$) (Fig. 4). The mRNA copy numbers for *IGHG1* were relatively high in horses 1, 3, and 4; a similar result was observed for *IGHG4/7* in horses 2 and 5 (statistical analyses were not performed because of the low power when dividing the leukemic horses in 2 groups). The *IGLC* copy number was higher ($P = 0.02$) in all affected horses than that in the controls. Horses 2 and 5 had *IGKC* copy number expression comparable to that of the control healthy horses ($P = 0.13$) (Fig. 4). The *IGLC/IGKC* copy number ratios for horses 1 through 5 were 21,063, 164, 9,898, 22,542, and 439, respectively, and the values for the 3 control healthy horses were 85, 68, and 142. Overall, the *IGLC/IGKC* copy number ratio was greater ($P = 0.03$) in affected horses than that in the control horses. The bone marrow and

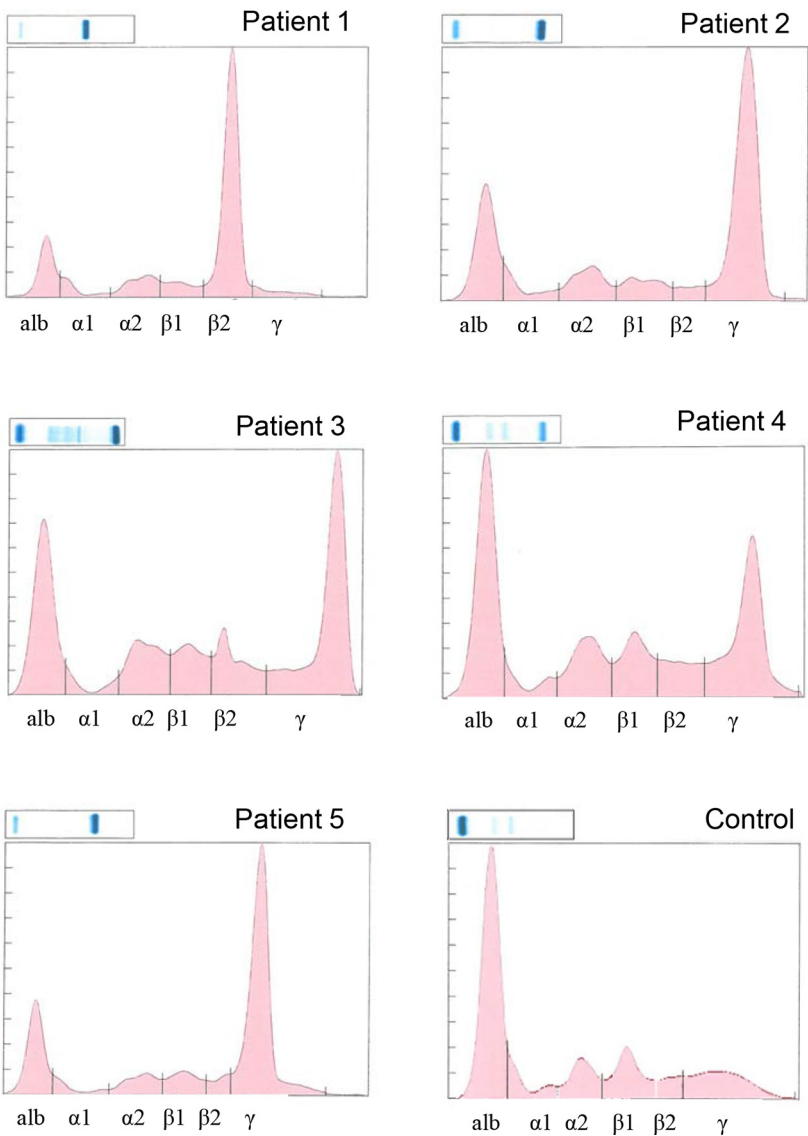


FIG 2 Electrophoretic profile of serum (or plasma) proteins of 5 equine patients with B cell neoplasm, characterized by leukemia and hyperproteinemia. Note the monoclonal peaks of globulins in the gamma region, except for horse 1, which is seen in the beta 2 region. Electrophoresis was performed with a plasma sample from horse 3 and shows a small peak (fibrinogen) in the beta 2 region. A serum sample from a control healthy horse is included for comparison.

mesenteric lymph node assay control samples from a healthy horse were positive for all genes in both RT-PCR and quantitative RT-PCR analyses (not shown).

Immunoglobulin spectratyping. Immunoglobulin spectratyp-

ing revealed one dominant peak for each affected horse, with amplicon lengths ranging from 420 to 439 bases in the individual horses (Fig. 5). For control healthy horses, ≥4 peaks were observed for each horse, ranging from 424 to 442 bases in 3-base intervals, and the

TABLE 5 Serum immunoglobulin concentration results

Affected horse or reference interval	Radial immunodiffusion results (mg/dl) for:			Turbidimetric assay results (mg/dl) for IgG	ELISA results (mg/dl) for:		
	IgA	IgM	IgG		IgG1 (IgGa)	IgG4/7 (IgGb)	IgG3/5 (IgGT)
1	NA ^a	25	800	5,084	11,025	189	115
2	NA	<25	>1,600	7,391	66	5,835	22
3	225	25	2,400	5,043	4,130	526	687
4	350	70	3,450	3,569	2,862	1,070	862
5	31	<25	>5,000	7,833	290	7,817	314
Reference interval	150–360	100–110	960–3,200	960–3,200	207–479	531–1,697	263–462

^a NA, not available.

% positive cells in the lymphocyte-gated area for:

Affected horse or reference interval	CD2	CD3	CD4	CD5	CD8	CD19-like	CD21	IgM	IgG1 (IgGa)	IgG4/7 (IgGb)	IgG3/5 (IgGT)	IgG6 (IgGc)	IgA	MHC-I	MHC-II	LFA-1
1	16.9	6.8	2.6	3.2	1.4	10.4	ND ^a	1.7	92.4	0.4	0.8	0.3	3.8	99.9	96.4	99.5
2	ND	11	5.3	6.4	9.6	2.6	ND	ND	2.1	87.7	1.2	ND	0.2	100	99.1	98.3
3	5.5	6.9	1.3	5.5	3.6	14.1	7	20.1	75.9	0.2	0.3	0.1	ND	100	99.1	99.9
4	2.7	1.1	2.3	3.3	0.7	0.2	1.5	0.3	98.1	0.2	0.7	2.4	ND	100	98.8	100
5	10.1	9.6	5.2	10.8	5.4	2.1	8.6	4.7	1.4	93.4	2.5	ND	ND	100	99.5	98.9
Reference interval	80.7–89.9	86.3–93.0	57.2–72.1	86.2–95.6	14.1–22.7	6.3–12.6	3.4–17.9	5.6–12.9	<2.0	<2.0	<2.0	<2.0	<2.0	99.9–99.9	84.6–98.9	99.7–99.9

amplicons were of approximately equivalent magnitude within a horse.

Equine IGHV domain sequencing. Immunoglobulin heavy-chain variable region sequences were amplified from peripheral blood leukocytes for all 5 affected horses, and ≥ 10 clones were sequenced per horse. For some horses, the sequences obtained were 99 to 100% identical (horses 2, 3, and 4), and for other horses (1 and 5), more than one sequence was obtained (Fig. 6). Using the 98% germ line identity criterion, sequence analysis of the IGHV domain indicated a high frequency of mutated genes, with the exception of clones P1.12 to P1.15, (horse 1) and P5.1, P5.4, P5.5, and P5.7 to P5.11 (horse 5).

The lymphoid neoplasms presented in this study manifested in middle-age to elderly horses as concurrent lymphadenopathy, mild to marked leukemia, and hyperglobulinemia due to monoclonal gammopathy of the IgG1 or IgG4/7 isotype. Peripheral blood leukocyte immunophenotyping revealed a markedly increased percentage of cells expressing surface IgG1 or IgG4/7 and concomitant T cell lymphopenia. The leukemic cells did not express the classic B cell markers CD19 and CD21, although two horses had low numbers of presumably normal CD19⁺ or CD21⁺ cells. At the mRNA level, the expression of the B cell markers *EBF1*, *PAX5*, and *CD19* was greater in leukocytes of affected horses than that in the control healthy horses, in contrast to the mRNA expression of the plasma cell marker *CD38*. In addition, high mRNA expression for *IGHG1* or *IGHG4/7* in leukocytes was consistent with their respective cell surface protein expression, secreted monoclonal gammopathy, and single dominant peak on Ig spectratyping. The *IGLC/IGKC* copy number ratio was greater in affected horses than that in the control horses, but 2 affected horses still expressed detectable levels of mRNA for *IGKC*. Sequence analysis of the IGHV domain indicated a high frequency of mutated Igs in comparison to the germ line. The expression of *IGHM* in horses 3 and 5 was not consistent with serum IgM concentrations, which were low overall.

Lymphocytosis of small to intermediate lymphocytes was the most consistent change in the hemogram. Lymphocytosis was mild in one horse and moderate to marked in the others. The lymphocytosis observed in these cases was attributed to leukemia due to aberrant phenotypes of the lymphocytes in blood. Mild anemia was attributed, in general, to the suppression of erythropoiesis from chronic disease. Although myelophthisis was not reported in the postmortem evaluation, horse 3 had infiltrates in the bone marrow, which may have contributed to decreased red blood cell production and the concurrent severe thrombocytopenia, with associated bleeding signs. Thrombocytopenia might also have been due to concurrent consumption due to severe infection based on an inflammatory leukogram or immune-mediated mechanisms not investigated antemortem (31, 32).

Monoclonal gammopathy was suspected in these equine patients due to excessive rouleaux formation in erythrocytes, moderate to severe hyperglobulinemia, and associated lymphocytosis. Monoclonal gammopathy was confirmed by protein electrophoresis (serum or plasma) and Ig spectratyping (blood cell) in all cases. In one patient, the monoclonal protein was observed in the beta 2 region; although most IgG proteins migrate to the gamma region, they can be found throughout the electrophoretic spectrum, such as in the beta region. Since urine samples were not

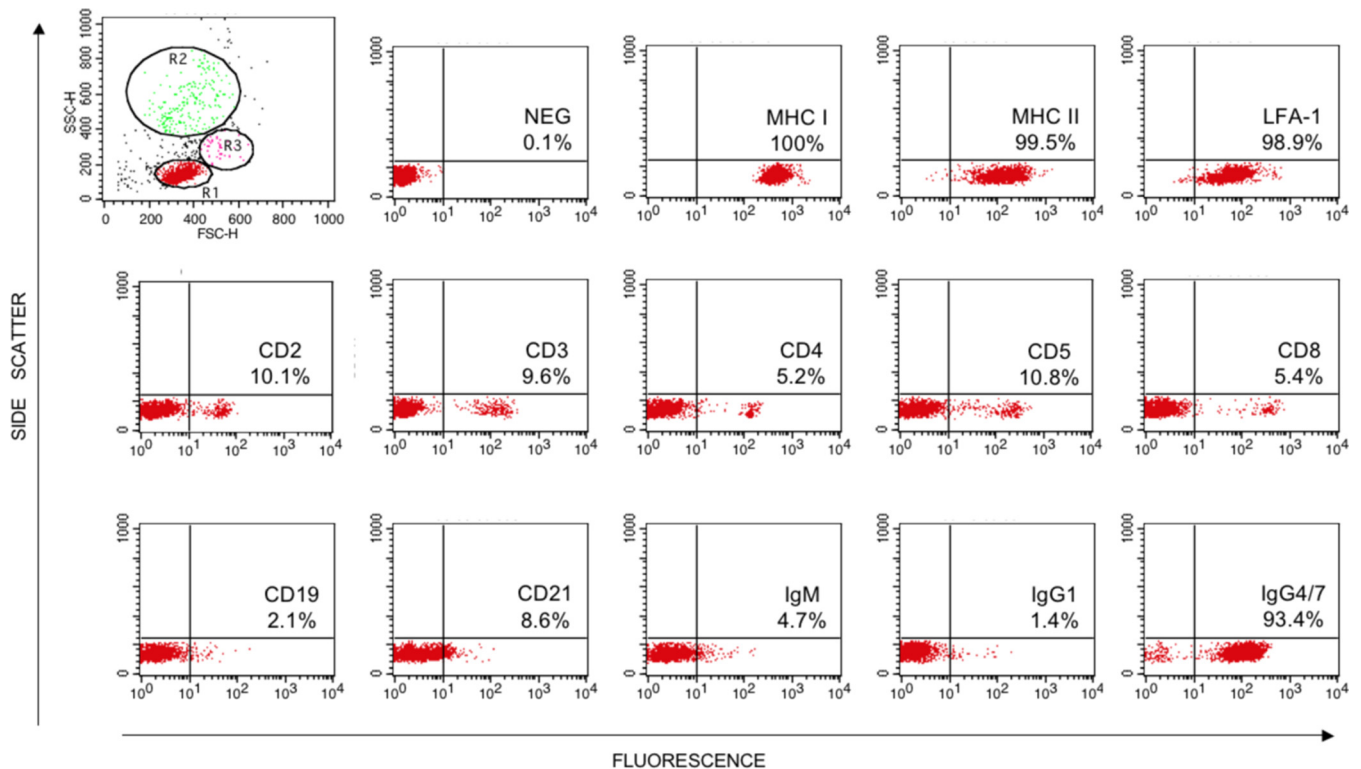


FIG 3 Flow cytometric dot plot analysis of peripheral blood immunophenotyping of a horse with leukemia. Peripheral blood immunophenotyping of horse 5 shows the distribution of leukocytes (SSC-H, cell granularity side scatter height; FSC-H, cell size forward scatter height) with the prevalence of lymphocytes (R1) in comparison to neutrophils (R2) and monocytes (R3). Dot plots of SSC versus fluorescence show the distribution (%) of positive cells within the lymphocyte-gated area (R1) for each of the markers tested. Neg, negative.

available for these horses, tests for the presence of monoclonal light chain in urine (i.e., urine electrophoresis and heat precipitation methods) were not performed. None of the horses submitted to necropsy had evidence of renal azotemia, despite having monoclonal gammopathy and neoplastic infiltrates in the kidneys. It is notable that the RID assay for the measurement of serum IgG was the least accurate method for detecting the monoclonal protein, yielding normal values in two horses despite markedly high concentrations of IgG measured by both ELISA and the immunoturbidimetric assay; these two assays were in closer agreement. This might be due to the insensitivity of the antibody in the RID kit to a potentially misfolded or mutated monoclonal IgG.

For this study, we performed mRNA analysis in leukocytes that contained a mixture of normal and leukemic cells. Despite the mixed nature of the cells in the preparation, the expression of signature B cell genes (*EBF1*, *PAX5*, and *CD19*) was greater in affected horses, likely reflecting the increased proportion of neoplastic B cells in the peripheral blood. Surprisingly, the increased *CD19* mRNA expression in the leukemic cells was not associated with expression of the protein on the cell surface. The magnitude of gene expression of *IGHG1* and *IGHG4/7* agreed with the magnitude of correspondent distribution of circulating cells (immunophenotyping) and serum IgG1 and IgG4/7 concentrations, respectively. With the marked expression of IgG on the cell surface, this might be explained by a tumor arising from a B cell in transition phase between a postgerminal center B cell and plasma cell, or perhaps an aberrant plasmablast or immature plasma cell. The high serum concentrations of secreted IgG antibodies indicated

that the neoplastic B cell clone had undergone isotype switching after encountering antigen and moved on to the antibody-secreting phase. The CD19 molecule is expressed during B cell differentiation commitment through antigen-independent and -dependent development but not after differentiation into plasma cells (33). Along with CD21 and CD81 coreceptors, CD19 assembles the B cell Ig receptor and regulates B cell activation upon antigen binding. In support of our findings, previous studies of CD19 in horses affected with monoclonal gammopathy have shown that leukemic B cells frequently lose surface expression of this marker, despite the persistence of IgG expression (7, 34). These B cell monoclonal antibodies also detected a small population of normal (not leukemic) cells in the samples of these equine patients, indicating their potential to detect the equine molecule when present. Due to the lack of appropriate equine conjugated-antibody reagents, confirmation of negative or low expression of CD19 and CD21 molecules in the IgG1 and IgG4/7 leukemic cells was not possible. The modest *CD38* mRNA expression, along with lymphoid features of the leukemic cells, suggests that the neoplastic clone had not fully differentiated into plasma cells. In human patients, surface expression of CD38 in B-CLL carries an unfavorable prognosis, since this protein is required for cell proliferation and survival (35). The lack of equine reagents for other markers used for the diagnosis of B and plasma cell neoplasms in human patients (e.g., CD20, CD23, CD27, CD138, and MUM-1) impairs further classification of the leukemic cells in horses at the protein level (36).

In human patients with B-CLL, there is usually only a single

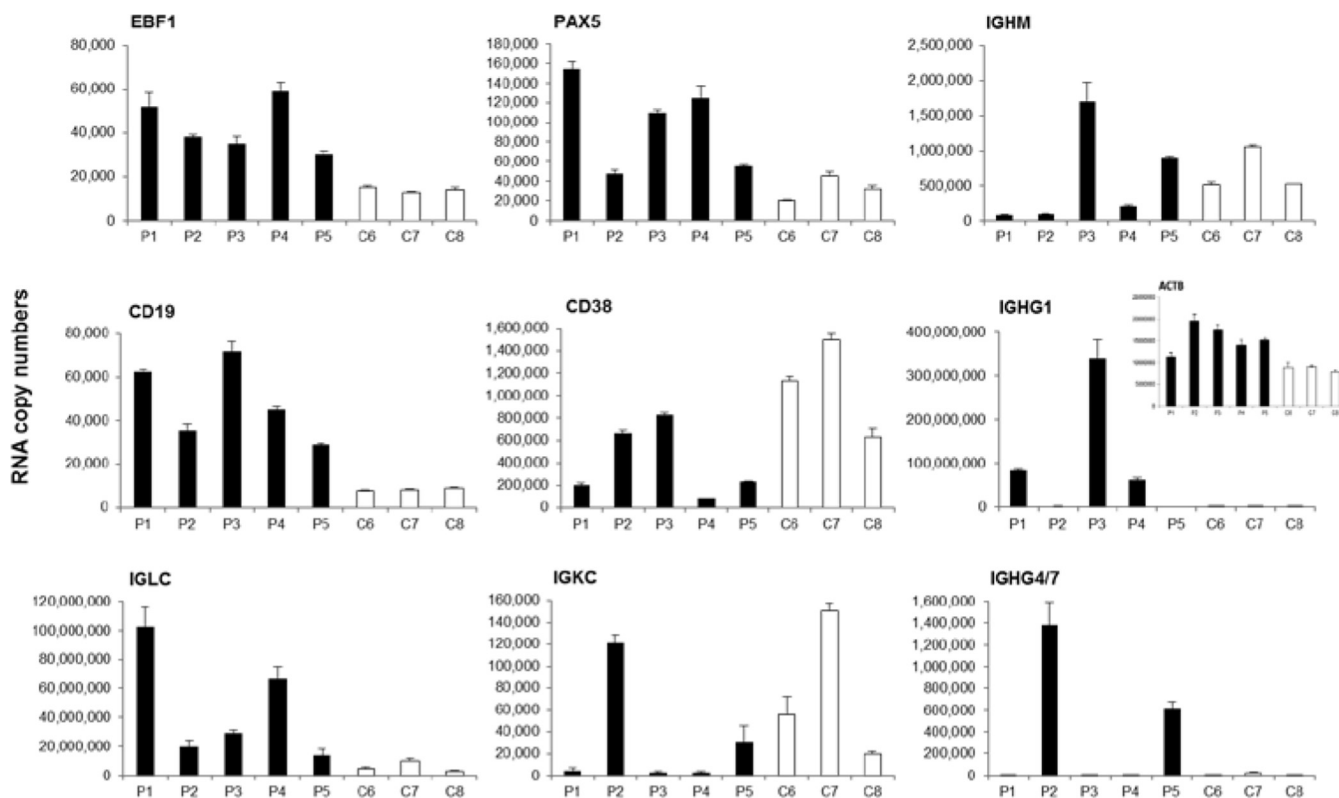


FIG 4 Quantitative gene expression (mean value $\pm$ standard error) of B cell signature genes in peripheral blood leukocytes isolated from horses with B cell neoplasm and leukemia and in healthy horses. The mRNA copy number of B cell signature genes was measured in triplicate using quantitative real-time RT-PCR and RNA extracted from isolated leukocytes of affected horses (horses P1 to P5; filled bars) and healthy horses (controls C6 to C8; open bars). The results were compared to serial dilutions of respective *in vitro*-transcribed RNA used for the generation of a quantitative standard curve. The expression of the housekeeping gene β -actin (ACTB) in the respective samples is shown as an inset. Bone marrow and mesenteric lymphoid tissues from a healthy horse were tested side by side as positive controls (not shown).

clone of cells that expresses one class of Ig light chain (kappa or lambda), which results in an altered kappa/lambda ratio when measured by flow cytometry (37). However, measurement of the kappa-lambda ratio by flow cytometry may fail to identify smaller clonal populations admixed with reactive polyclonal B cells, and mRNA expression may increase the sensitivity for detecting the neoplastic clone. As there are no reliable monoclonal antibodies available to measure equine kappa and lambda light chains using flow cytometry, we measured gene expression in leukocytes. Overall, affected horses had a greater *IGLC/IGKC* copy number ratio than that of the control healthy horses, indicating the predominance of the lambda chain in leukemic cells; this result was somewhat expected, because horses produce Ig with a relative abundance of lambda (96%) in comparison to kappa (4%) light chains under normal conditions (38). Nevertheless, 2 horses had comparable *IGKC* expression to that of the control horse and relatively higher expression than that of the other affected horses, suggesting either expression of both light chains by leukemic cells or reflecting the presence of a high number of normal B cells expressing the kappa light chain.

The combination of flow cytometric and molecular techniques has expanded our ability to diagnose and better understand the pathophysiology of lymphoid neoplasms. Furthermore, these techniques can readily be performed antemortem on blood samples or tissue aspirates versus relying on necropsy and histologic

evaluation of formalin-fixed tissue for a definitive diagnosis. Here, we provide the first report of two molecular techniques that confirm the diagnosis of mature B cell lymphoma/leukemia in horses. Assessments of B cell IGHV clonality and sequencing have widely been used in human patients and infrequently in dogs with lymphoid neoplasia (leukemia and lymphoma) (5, 39, 40). Rearrangement of the IGHV region and expression of a functional B cell receptor occur during B cell development in the bone marrow, and mutations may follow during antigen-dependent development (41). Human patients with unmutated IGHV genes ($>98\%$ homology with the germ line) have a poorer prognosis than that of patients with mutated IGHV genes ($<98\%$ homology) (5). The lack of chromosomal translocations in samples from human patients suggests that B cell leukemia originates from the oncogenic transformation of a germinal center-derived B cell, i.e., after a T cell-dependent immune response, somatic IGHV hypermutation, and Ig isotype switching (42). Alternatively, marginal-zone B cells involved in T cell-independent immune responses become transformed with unmutated IGHV receptors, or transformation occurs before B cells enter the germinal centers, although mutated cases have also been reported (5). The expression of CD5 has been used to identify marginal-zone B cells (43). Our data showed that the equine leukemic cells were negative for the CD5 marker, and a majority had a mutated IGHV domain, suggesting a B cell neoplasm of postgerminal center cells that had undergone somatic

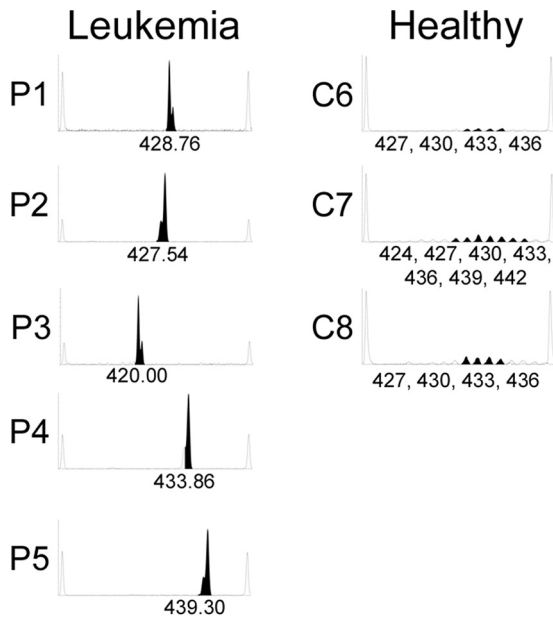


FIG 5 Immunoglobulin spectratyping of peripheral blood leukocytes isolated from horses with B cell neoplasm and leukemia and from healthy horses. Immunoglobulin heavy-chain transcripts were amplified from isolated leukocytes of affected horses (P1 to P5) and healthy horses (controls C6 to C8). Spectratypes (black peaks) of the fluorescently labeled amplicons were visualized on a genetic analyzer with a size standard (white peaks at 400 and 450 bases shown). Amplicon lengths are shown. Leukemic samples generated one amplicon, indicating monoclonality, and samples from control healthy horses generated multiple amplicons, indicating a polyclonal population.

hypermutation and isotype switching in the peripheral lymphoid tissues.

Immunoglobulin spectratyping was a novel approach to measure monoclonality; it provided a graphic representation of the overall Ig repertoire based on the distribution of CDR3 lengths and revealed one dominant peak for affected horses in contrast to control horses (44). Ig gene rearrangement is imprecise; therefore, CDR3 lengths are variable in nucleotide length. Spectratyping assesses CDR3 length distribution, because length heterogeneity is expected to represent overall sequence diversity. In the assay presented here, Ig transcripts are amplified by conserved primers in fixed positions, and the resulting products directly reflect the CDR3 length distribution of the template. The number and distribution of peaks observed, or spectratyping, were consistent with the IGHV sequences presented here and with previous IGHV sequence analyses (18). Although the IGHV sequence analysis provided additional detail, spectratyping was sufficient to discriminate between leukemic and healthy samples. In this study, spectratyping had 100% agreement with protein and molecular tests performed in leukocytes, bringing the potential for antemortem diagnostic application of clinical cases, including nonsecretory B cell leukemias (not diagnosed using protein electrophoresis), cavity fluids, or solid-tumor aspirates.

The horses in this report had both extramedullary infiltrates (such as lymphadenopathy) and lymphocytosis, indicating concurrent leukemia. In human patients, CLL is a lymphoproliferative disorder characterized by monoclonal B lymphocytosis; the clinical signs are heterogeneous, ranging from asymptomatic and

prolonged survival to the presence of lymphadenopathy, hepatosplenomegaly, autoimmune cytopenias, weight loss, fatigue, and poor outcome (2). SLL is characterized by lymphadenopathy, hepatosplenomegaly, and tumoral cell invasions; monoclonal B cells can be in circulation but in lower number than in CLL. SLL is considered the tissue equivalent (nonleukemic) of CLL. The mild differences in clinical manifestations can be associated with their distinct expression of chemokine receptors, integrins, and genetic abnormalities, although CLL and SLL in humans do not seem to differ morphologically and at the level of cell surface molecules: both types of tumors can express CD2, CD3, CD5, CD19, CD21, and CD23.

Another unsolved observation in this study was the fact that patient 3 was diagnosed postmortem with a T-cell-rich large B cell lymphoma, which is characterized by large neoplastic B cells with a background population of presumably polyclonal small T cells. However, the majority of circulating neoplastic cells in this horse were small to intermediate in size and lacked T or B cell markers, similar to the other cases reported here. In human patients, CLL may show an increase over time in cell size and proliferative activity in lymph nodes and bone marrow. Indeed, 2 to 8% of human patients with CLL/SLL develop diffuse large B cell lymphoma, with an aggressive clinical course and poor prognosis; the molecular mechanisms associated with neoplasia transformation are under investigation (45). In addition, human patients with follicular lymphoma may have lymphocytosis of the small B cells, while the tumor in the lymph node can be composed of larger cells (46). The lymph node tissues of these patients were not available for spectratyping and sequencing, which potentially might have helped solve the origin of and relationship with the leukemic cells.

In addition to genetic and epigenetic abnormalities, lymphomagenesis of mature B cell neoplasms involves the sustained activation of self- or nonself-antigen-driven B cell receptor (BCR) clonal proliferation and impaired apoptosis (47). The active proliferation state creates an opportunity for the use of therapeutic agents that target the BCR signaling pathway, including those that inhibit the function of spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK), phosphatidylinositol 3-kinases (PI3K), and B-cell lymphoma 2 (BCL-2) (48). In human patients, current treatment options include chemotherapy (e.g., fludarabine and cyclophosphamide), radiation therapy, immunotoxins (e.g., rituximab), and bone marrow transplantation. In dogs, successful treatment has been reported with melphalan and prednisolone (49). In horses, treatment of lymphoid neoplasms is rarely performed due to poor characterization of neoplastic cells, high costs per body weight, and poor results, and it was not an economic possibility in these patients.

In summary, the B cell neoplasms presented here were characterized by small to intermediate lymphocytes with concurrent tissue and blood involvement and monoclonal gammopathy of IgG isotypes. The leukemic cells expressed markers compatible with a developmental transition between a B cell and a plasma cell stage, including IgG⁺ CD19⁺, and predominant mutated IGHV. Our study brings a systematic and comprehensive approach that uses molecular testing to complement previously used cytologic and histologic protein testing, enabling advanced diagnosis and classification of B cell neoplasia in these patients. Future studies using this approach may help with identifying new therapeutics and prognostic markers for these tumors in horses.

Consensus	WFFLFVAAPTCLVSLQVQLKESGPGLVKPSQTLISLCTVSGLSLSSXXVGNVRQAPGKGLXGVXI---XXXGSXXNPA--LKSRAITKDTSKSQVYLTINSLTSEDTAVVYCGA-----XXXXXXXXXXBYWGQGLIVTSS
P1A6	NA.....S.....F..W.....DASA.AW.S.G.....S.....T.....-WIGS--YYSNDCTP
P1.6	NT.....W.AD.....DGDE.AY.....R..I.....S.....G.....R.....RSATTWYSSFYIN.....M.....
P1.5	NTI.....F..VL.....HGYTPN.....R--R..F.....A..GLL.....TV.ED.....-GEGDSWGNVYG.....T.....
P1.8	NTI.....F..VL.....HGYTPN.....R--R..F.....A..GLL.....TV.ED.....-GEGDSWGNVYG.....T.....
P1A7	T.NT.....F..Y.....YGA.AS.M.....-G--QFGGDYSYV--SGLDYGVN.....
P1.7	Y.....Y.....F..A.....RSS..TN.....-G--VYGGGAFFYSYSSYSAII.....
P1.3	V.....T.V.....RID.A.....VF..AV--WGQV.AN.....S.....M.I.....A.....H.....F.....GVVPGFGYIGIEVTSYSGINH.....
P1.2	YT.....R.E.....RLSS.....A.E..VDWNI.....I..S..S..WI.F.....QNGDRIT.....KID.QY.....T.....S.QE.....V.....-WFGASGERHNIILN.....
P1.12	V.ASG.S.EG..EQ..E.K..GSSVKIS.KA..YTFT.YA.H.....N..I.WM.S.-Y-AYDDTS.A.K--FQG.VTM.A.K.T.TT.ME.S.....-TTKAAMLKTNEID.....T.....
P1.14	V.ASG.S.EG..EQ..E.K..GSSVKIS.KA..YTFT.YA.H.....N..I.WM.S.-Y-AYDDTS.A.K--FQG.VTM.A.K.T.TT.ME.S.....-TTKAAMLKTNEID.....T.....
P1.15	V.ASG.S.EG..EQ..E.K..GSSVKIS.KA..YTFT.YA.H.....N..I.WM.S.-Y-AYDDTS.A.K--FQG.VTM.A.K.T.TT.ME.S.....-TTKAAMLKTNEID.....T.....
P1.13	V.....V.ASG.S.EG..EQ..E.K..GSSVKIS.KA..YTFT.YA.H.....N..I.WM.S.-Y-AYDDTS.A.K--FQG.VTM.A.K.T.TT.ME.S.....-TTKAAMLKTNEID.....T.....
P2B6	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2B12	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.1	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.2	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.3	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.4	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.5	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.6	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.7	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.8	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.9	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P2.10	N.YG.....F..D.--LSS..AN.....-WYSS--YYELGVN.....
P3.1	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.2	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.4	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.5	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.6	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.7	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.8	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.9	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.10	K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P3.3	L.K.....F..N.YD.....Y..V.....YGA.AN.....-RYDLDDIMVYD.....G.....-RYDLDDIMVYD.....
P4.1	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.2	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.3	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.4	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.5	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.6	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.7	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.8	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.9	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P4.10	P.....S.A.....F..GV--GSD.LTRH.....-V.....-GSYSDLYYVSTIT.....
P5.1	W.....NA.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.4	W.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.7	W.....NA.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.8	W.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.9	W.....NA.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.10	W.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.11	W.....NA.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.5	W.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.3	W.....NA.....W..V.--YGSE.TY.....-G.....-FIGYSRYVNDYGINV.....
P5.2	P.....D.....D.NAI.....V.....KI..D.--MKS.RENIH.D--E.....-FIGYSRYVNDYGINV.....

FIG 6 Sequencing of immunoglobulin IGHV domain in peripheral blood leukocytes isolated from horses with B cell neoplasm and leukemia. The three complementarity-determining regions are underlined. Residues identical to the leukemic immunoglobulin VDJ consensus sequence are represented by dots, and dashes represent gaps inserted to maximize the alignment. Using the 98% homology with the germ line criterion, the sequence analysis of the IGHV domain indicated a prevalence of mutated genes, with the exception of clones 1.12 to 1.15 (horse P1), 5.1, 5.4, 5.5, and 5.7 to 5.11 (horse P5).

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