Case Report
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Infection with Panola Mountain *Ehrlichia* sp. in a Dog with Atypical Lymphocytes and Clonal T-Cell Expansion

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Case Report

An 11-year-old, castrated male Scottish Terrier from Raleigh, NC that lived predominantly indoors and had no travel history was referred to the North Carolina State University Veterinary Health Complex (NCSU-VHC) in October 2012 for routine reevaluation of hepatobiliary disease. The dog's previous 3-year medical history included biliary mucocele (2010); neutrophilic hepatitis (2011); recurrent *Escherichia coli* urinary tract infections, esophageal dysmotility, aspiration pneumonia, and transient thrombocytopenia (2012); and food responsive enteropathy (2009–2012). All of these medical problems were well controlled at the time of examination and no clinical abnormalities were reported by the dog's owners. In the months before the present examination, ticks occasionally were noted and fleas were commonly found on the dog despite reported use of preventive therapies.

On physical examination, the dog was obese (body condition score, 7 out of 9) and had mild hepatomegaly, both of which had been present for more than a year. Notable CBC findings included mild thrombocytopenia (platelet count, 143,000/μL; reference interval [RI], 190,000–468,000/μL) and an increased number of atypical lymphocytes (1,773/μL), with normal appearing lymphocytes within the laboratory reference range (1,854/μL; RI, 594–3,305/μL) and an otherwise normal differential cell count. A review of the blood smear by a pathologist identified a population of immature lymphocytes with angular nuclei and cell shape, multiple nucleoli, and deeply basophilic, vacuolated cytoplasm that had a tendency to mold into surrounding cells (Fig 1). These morphologic abnormalities raised the suspicion of possible lymphoid neoplasia, although these changes can be seen secondary to reactive processes such as chronic inflammatory or infectious diseases. Serum biochemical abnormalities included an increase in alkaline phosphatase activity (ALP; 817 IU/L; RI, 16–140 IU/L) and alanine aminotransferase activity (ALT; 60 IU/L; RI, 12–54). During the previous 12-month period, ALT activity varied between normal and 80 IU/L and ALP activity varied between 359 and 534 IU/L. Because these hematological and serum biochemical abnormalities were present 2 weeks later, abdominal ultrasound examination was repeated and identified mottled splenic parenchyma

Abbreviations:

NCSU-VHC	North Carolina State University Veterinary Health Complex
ALP	alkaline phosphatase activity
ALT	alanine aminotransferase activity
PCR	polymerase chain reaction
PARR	antigen receptor rearrangements
EDTA	ethylenediamine tetraacetic acid
NCSU-CVM-VBDDL	NCSU-CVM Vector Borne Diseases Diagnostic Laboratory
BAPGM	*Bartonella* alpha-proteobacteria growth medium
IFA	indirect immunofluorescent antibody
PME	Panola Mountain *Ehrlichia*

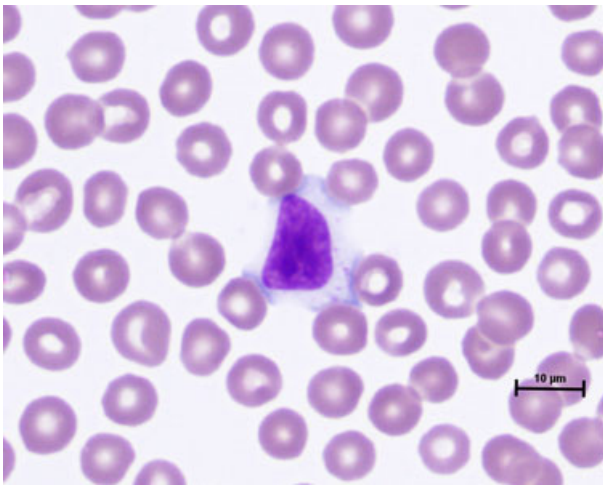

Fig 1. Peripheral blood from a dog with clonal T-cell expansion. Atypical lymphocytes display large angular nuclei with abundant pale basophilic cytoplasm that frequently contains several small eosinophilic cytoplasmic granules. Wright–Giemsa stain with 100× magnification.

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and several previously identified changes including hyperechoic nodular hepatomegaly; mild dystrophic mineralization of the spleen, liver, kidneys, and prostate; and, a thickened urinary bladder apex. Splenic cytology identified a mixed lymphoid population and mild extramedullary hematopoiesis. Liver cytology identified normal hepatocytes and an expansion of intermediate lymphocytes, raising concern for lymphoma. Cytology of a palpably normal popliteal lymph node identified expansion of intermediate to large lymphocytes, most consistent with lymphoma (Fig 2). T-cell clonality was documented in a liver aspirate after submission to NCSU-CVM Clinical Immunology Laboratory for polymerase chain reaction (PCR) to identify antigen receptor rearrangements (PARR). Aseptically collected ethylenediamine tetraacetic acid (EDTA)-anticoagulated whole blood and serum were submitted to the NCSU-CVM Vector Borne Diseases Diagnostic Laboratory (VBDDL) for a vector-borne pathogen serology panel and *Bartonella* alpha-Proteobacteria Growth Medium (BAPGM) enrichment blood culture PCR platform. The dog was seronegative for antibodies to *Anaplasma phagocytophilum, Anaplasma platys,* and *Borrelia burgdorferi* and for *Dirofilaria immitis* antigen using a commercial enzyme-linked immunosorbent assay based kit (SNAP® 4Dx® Plusa); the dog was seronegative for antibodies to *Babesia canis, Babesia gibsoni, Bartonella henselae,* and *Bartonella vinsonii* subsp. *berkhoffii*, using indirect immunofluorescent antibody (IFA) testing. By IFA, the dog was seroreactive to *Rickettsia rickettsii* (1:64; laboratory cut-off value, 1:64) and *Ehrlichia canis* (1:1028; laboratory cut-off value, 1:64) antigens, but seronegative to *Ehrlichia* spp. peptides in the SNAP 4Dx Plus[a] kit, which is known to detect antibodies against *E. canis, E. chaffeensis,* and *E. ewingii*. *Bartonella* enrichment blood culture and PCR were negative. Nine months before this presentation, the dog was seronegative in the NCSU-CVM-VBDDL to the vector-borne pathogens tested above.

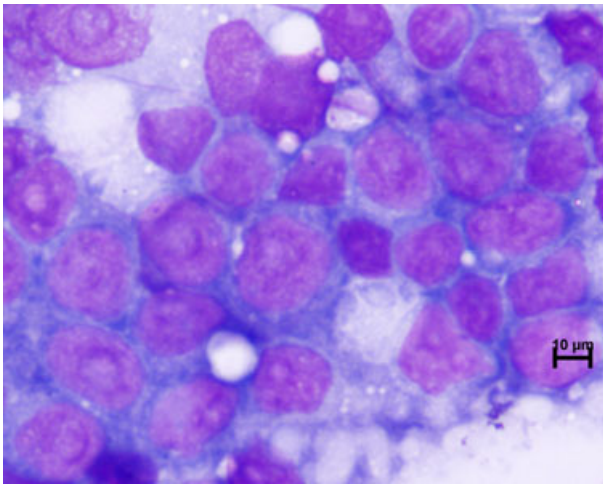

Fig 2. Fine needle aspirate of lymph node from a dog with clonal T-cell expansion. Intermediate to large lymphocytes with round to oval nuclei, occasional prominent nucleoli or several small chromacenters and a narrow rim of basophilic cytoplasm outnumber small lymphocytes. Wright–Giemsastain with 100× magnification.

Because of the discrepancy between the *E. canis* IFA and SNAP 4Dx Plus[a] test results, *Ehrlichia* spp. PCR was performed on DNA extracted from the dog's EDTA-anticoagulated whole blood using a previously described 16S ribosomal RNA PCR assay.[1] A 351 base pair portion of the 16S rRNA gene was amplified, sequenced, and investigated in the NCBI GenBank nucleotide database. The partial 16S rRNA gene identified infection with the Panola Mountain *Ehrlichia* sp. (PME) with 100% coverage (331 bp) and 100% identity to PME 16S ribosomal RNA gene (DQ324367.1). To confirm these results, alternative PME genes, including *gltA* and *map1* were targeted. Furthermore, *sodb*, a gene not previously sequenced for PME, was amplified using newly developed primers designed to amplify *Ehrlichia* spp. *sodb* genes. Primers designed to amplify a 311 bp portion of PME *gltA* (EPM-gltA-Forward, 5′-CGTGTTTTTCTGCCTTAGCTGCAC and EPM-gltA-Reverse, 5′-CGGCCCAGAAGAACC TGTC A) based on a published PME *gltA* sequence (DQ363995.1) and a second set of primers designed to target a 480 bp portion of PME *map1* (EPM-map1-Forward-3, 5′-CGAGAGCCAACGTTTACAT and EPM-map1-Reverse-2, 5′-GTACCAATACCTGCACATAC) based on published PME *map1* sequences (DQ324368.1, EU272373.1 and EU272355.1) were used. Primers designed to amplify a 300 bp portion of *sodb* (sodb-Forward, 5′-TTTAATAATGCTGGTCAAGTATGGA ATCAT and sodb-Reverse, 5′-AAGCGTGTTCCCA TACATCCATAG) based on published *Ehrlichia* spp. *sodb* sequences (AF392615.1, CP000107.1) were used. Reactions contained 5 µL of DNA extract, 12.5 µL of MyTaqHS-2X[b], 0.125 µM (or 0.25 µM for *sodb* primers) of each primer[c] and RNAse-free, molecular-grade water to a final volume of 25 µL. All reactions were performed in a thermocycler[d] with an aluminum block under the following conditions, with respective primer annealing temperatures specified: initial temperature at 94°C for 3 minutes, 55 cycles consisting of denaturation at 94°C for 10 seconds, annealing at 68°C (*gltA*), 62°C (*map1*), 58°C (*sodb*) for 10 seconds, extension at 72°C for 15 seconds, and a final extension at 72°C for 30 seconds. Negative controls (RNAse-free, molecular-grade water and uninfected canine genomic DNA) were included in all assays. Amplified PCR products were sequenced directly[e] and alignments were made with GenBank sequences using AlignX software.[f] Sequence identities for the partial *gltA* and *map1* genes are as follows, all with 100% coverage: *gltA*, 100% similar (269 bp) to PME partial *gltA* gene from an infected goat (DQ363995.1) and *Amblyomma americanum* tick (EU272374.1); *map1*, 100% similar (460 bp) to PME partial *map1* gene from an infected goat (DQ324368.1) and *A. americanum* tick (EU272373.1). Before this study, there was no sequence for PME *sodb* deposited in GenBank. The highest sequence identities assigned by BLAST[g] for the partial gene amplified using *Ehrlichia* spp. *sodb* primers are as follows, all with 100% coverage: *sodb*, 89%

(264/295 bp) similar to multiple strains of *E. ruminantium sodb* gene, with the highest Max scores reported for Senegal (DQ647026.1), Pokoase (DQ647024.1), and Kumm1 (DQ647023.1) strains, 83% (245/295 bp) similar to *E. canis* strain Jake *sodb* (CP000107.1) and 80% (236/295 bp) similar to *E. chaffeensis* strain Arkansas *sodb* (AF392615.1). The PME partial *sodb* gene sequence was submitted to Genbank (Accession number KC702804). Primer sets specific for *Rickettsia ompA, E. canis p30*, *E. ewingii p28*, and *E. chaffeensis p28* did not amplify DNA from the PME-infected blood sample, suggesting this dog was not likely coinfected with *Rickettsia* or other *Ehrlichia* species. Retrospective PCRs using DNA extracted from predoxycycline splenic cytologic smears were negative (16S rRNA, *gltA, map1*, *sodb, E. canis p30*, *E. ewingii p28*, and *E. chaffeensis p28)* for pathogen DNA.

Treatment for suspected lymphoma was not initiated because of the possibility that lymphocytosis and other lymphocytic abnormalities were due to a reactive process secondary to ehrlichiosis.[2–4] Treatment with doxycycline (approximately 5 mg/kg PO q12h) was commenced for 30 days. After starting doxycycline treatment, occasional vomiting was noted. Resolution of the thrombocytopenia and lymphocytosis occurred 1 week after starting the treatment. ALP and ALT activities remained increased at 1,989 IU/L and 119 IU/L, respectively. One week after completion of doxycycline, repeat aspiration cytology identified a mixed lymphoid population with expansion of intermediate lymphocytes and mild extramedullary hematopoiesis within the spleen, a mixed lymphoid population within the popliteal lymph node, and an expansion of intermediate lymphocytes and mild vacuolar change within the liver. Flow cytometry of the liver showed a population of large, granular cells with positive intracellular staining for CD3, consistent with T-cell lymphoma. When cytology was repeated again 4 weeks after completion of doxycycline, there was resolution of the abnormal lymphoid population in liver and lymph nodes, but there were increased numbers of lymphoblasts and mild persistent extramedullary hematopoiesis within the spleen (Fig 3). ALP and ALT activities had decreased to 1,352 IU/L and 66 IU/L, respectively. Throughout the 3-month time period described in this case report, the owners reported no clinical abnormalities other than occasional gastrointestinal signs, but felt in retrospect that the dog may have been slightly lethargic as they reported it was more energetic after completion of the doxycycline treatment regimen. Furthermore, 5 subsequent CBCs performed over the next 6 months remained normal.

EDTA-anticoagulated whole blood and convalescent serum samples collected from the dog approximately 2 weeks after starting doxycycline treatment were PCR negative for PME (16S rRNA, *gltA, map1* and *sodb*) and the SNAP 4Dx Plus was positive for anti-*Ehrlichia* spp. antibodies, respectively. Convalescent serum, collected approximately 5 weeks after completion of antibiotic treatment, was minimally reactive (1:32) to *R. rickettsii* antigens, whereas the dog remained *E. canis*

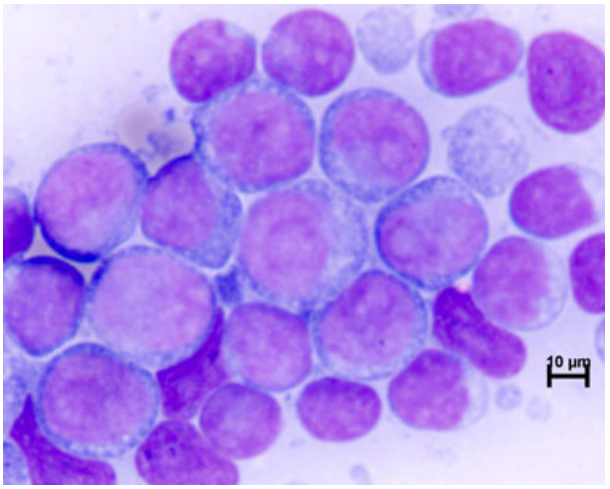

Fig 3. Fine needle aspirate of spleen from a dog with clonal T-cell expansion taken 4 weeks after completion of doxycycline treatment. Increased numbers of large immature lymphocytes with round nuclei, often large prominent nucleoli, fine chromatin pattern, and a rim of dark basophilic cytoplasm are present. Wright–Giemsa stain with 100× magnification.

seroreactive by both IFA (1:512) and SNAP 4Dx Plus[a] (weak *Ehrlichia* spp. positive). EDTA whole blood collected at this time remained PCR negative (16S rRNA, *gltA, map1* and *sodb*) for PME.

In this report, we provide molecular evidence of PME in a thrombocytopenic dog with abnormal lymphocytosis and clonal T-cell expansion. Treatment with doxycycline resulted in resolution of thrombocytopenia, abnormal lymphocytosis, and abnormal lymphoid cells in liver and lymph nodes, supporting a potential role for PME as a cause of host immune dysregulation. These findings also support a potential pathogenic role for PME as a cause of thrombocytopenia in dogs from the United States. Cytopathology and flow cytometry in this case identified a large number of atypical lymphocytes in the peripheral blood and in hepatic and splenic tissue aspirates with a high number of intermediate lymphocytes, granular CD3+ cells, and clonal T-cell expansion. Intracellular infections that induce cell-mediated immunity can cause cytological changes similar to malignancy.[5,6] A study characterizing peripheral blood smears in human ehrlichiosis patients infected with either *E. chaffeensis* or *E. ewingii* documented prominent large granular lymphocytes with atypical, folded, hyperchromatic nuclei that might be confused with neoplastic NK or NK-like T-cells.[6] Immunophenotypes compared between dogs with naturally acquired canine monocytic ehrlichiosis (CME) and healthy dogs found that dogs with CME had higher relative numbers of CD3+ T-cells in peripheral blood than did healthy dogs.[7] Additional studies found that dogs experimentally infected with *E. canis* had transitory CD8+ lymphocytosis in both peripheral blood and lymph nodes.[8,9] Additional studies describe clinically ill, *E. canis*-seropositive dogs with an increasing percentage of peripheral blood CD8+ lymphocytes.[3,10] PARR results from

the dog in this case demonstrated clonal T-cell expansion, typically associated with malignancy. At least 2 reports, however, describe this phenomenon in dogs with *E. canis* infections.[2,4] In a previous report, an *E. canis*-seropositive dog with pancytopenia and clonal T-cell expansion was treated with doxycycline, which resolved the pancytopenia and the dog remained healthy 2 years later.[2] Further investigation is needed to determine if expanded T-cells in *E. canis* infections are transitory or potentially could develop into lymphoid malignancy, particularly in association with chronic undiagnosed infections. As current evidence suggests, a role for *Ehrlichia* spp. in immune dysregulation is likely, but the extent may be subject to host factors, species and strain virulence, as well as phase of the disease. CME has acute, subclinical, and chronic phases representing different infection durations and variations in clinical disease manifestations. One recent study, however, found no statistically significant differences among CD3+, CD8+, and, CD4+ cells in peripheral blood samples from dogs with clinical or subclinical CME.[11] Signs of lymphoid malignancy continue to be monitored in the dog of this report. Resolution of the immunologic abnormalities noted in liver, lymph node, and blood after doxycycline treatment, however, strongly supports an infectious etiology.

To the authors' knowledge, this is the first report of PME infection in a dog. Genetically and antigenically similar to *E. ruminantium,* PME was first identified by PCR in a goat experimentally infested with *A. americanum* ticks collected from Panola Mountain State Park, Georgia.[12] Goats infected with PME developed serous nasal discharge and febrile illness with hematologic changes consisting of decreased ALP activities and neutropenia; rare morulae in mononuclear cells were identified in 1 goat.[12,13] PME also was detected by PCR in whole blood from a man in Atlanta, GA who developed myalgia after being bitten by a nymphal *A. americanum* tick.[14] No hematologic abnormalities were reported, and clinical signs resolved after doxycycline treatment. The role, if any, of PME in the overall pathogenesis of the various disease manifestations described in the dog of this report over its 3 year history of illness is impossible to assess. At the time PME was identified, the dog was asymptomatic and hematological abnormalities were limited to thrombocytopenia, increased numbers of atypical lymphocytes, and progressively increasing ALP activity (after initiation of doxycycline). The most notable characteristics of illness that potentially were related to PME infection in this dog included thrombocytopenia, cytological changes in the liver, lymph node and spleen, and clonal T-cell expansion. After treatment with doxycycline, sequential resolution of most of these abnormalities occurred. It is not clear when the dog became infected with PME, but serum screened for exposure to vector-borne pathogens 8 months earlier was negative, and the dog's owners reported flea and tick exposure over the previous 6-month time period. PME exposure presumably occurred in central NC because the dog had no travel history outside of the state. This is not a surprising finding, given documentation of PME in *A. americanum* from the eastern United States, with PME-positive ticks detected in FL, GA, KY, NJ, and NY.[15] In addition, deer from AR, NC, and VA were PCR positive for PME and were shown to be competent reservoirs for the pathogen.[16] Documentation of PME in this dog supports the possibility that this tick-borne organism may represent an unrecognized human or ruminant pathogen in NC and surrounding states.

Currently, serological diagnostics specific for PME are not available. Serum from goats infected with PME was weakly IFA seropositive to *E. chaffeensis* and seropositive by ELISA to the MAP1 protein from *E. ruminantium*.[12,13] Serum from the dog of this report reacted strongly with *E. canis* antigen by IFA, but less strongly with the synthetic antigens used in a commercial ELISA (SNAP 4Dx Plus). Antibodies to other *Ehrlichia* spp. such as *E. chaffeensis* have been shown to cross-react with *E. canis* antigens, and it is likely that the reactivity from this PME-infected dog also represented a cross-reaction.[17] Whereas *E. canis*, *E. ewingii,* and *E. chaffeensis* were not detected by PCR, we cannot rule out the possibility that seroreactivity was because of previous exposure with one of these pathogens.

In addition to highlighting the emergence of vector-borne pathogens in a novel host species, the findings in this case report underscore the challenges faced in diagnosing canine lymphocytic malignancies and reinforce the need to better understand the immunopathology of canine ehrlichiosis, which can be caused by *E. canis, E. chaffeensis, E. ewingii, E. muris,* and PME in dogs in North America. Documentation of abnormal or expanded lymphocyte populations in the blood or tissues of dogs should prompt diagnostic consideration of infection with an intracellular pathogen, including *Ehrlichia* spp.

Footnotes

[a] IDEXX Laboratories, Westbrook, ME
[b] Bioline, USA cat: BIO-25046, Taunton, MA
[c] Sigma–Aldrich, The Woodlands, TX
[d] Eppendorf Mastercycler-EPgradient, Westbury, NY
[e] GENEWIZ, Inc. Research Triangle Park, NC
[f] Vector NTI Suite 6.0, InforMax, Inc, Bethesda, MD
[g] NCBI, Bethesda, MD

Acknowledgment

Conflict of Interest: Authors disclose no conflict of interest.

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