Received: 9 January 2018 | Revised: 25 May 2018 | Accepted: 11 June 2018

DOI: 10.1111/vcp.12690

CASE REPORT

WILEY Veterinary Clinical Pathology
An International Journal of Laboratory Medicine

Molecular identification of *Ehrlichia ewingii* in a polyarthritic Texas dog

Joseph J. Modarelli[1,2] | Mindy M. Borst[1] | Julie Piccione[1] | Maria D. Esteve-Gasent[2]

[1]Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, Texas

[2]Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, College Station, Texas

Correspondence
J. Piccione, Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX.
Email: jpiccione@tvmdl.tamu.edu

Funding information
Texas AgriLife Research, Grant/Award Number: TEXV 6579 (ProkectI-9524)

Abstract

An 8-year-old, neutered male, Golden Retriever presented for bilateral carpal joint effusion. A complete blood count revealed mild leukopenia and marked thrombocytopenia. Samples were sent to the Texas A&M Veterinary Medical Diagnostic Laboratory for blood smear review and serologic testing for tick-borne diseases. Numerous morulae were observed within neutrophils, and antibodies against *Ehrlichia canis* were detected at a 1:512 dilution via the indirect fluorescent antibody (IFA) test. As neutrophilic morulae are morphologically indistinguishable between *Ehrlichia ewingii* and *Anaplasma phagocytophilum*, and genus-wide cross-reactivity is possible with serologic testing, additional molecular testing was performed. Quantitative real-time polymerase chain reaction (qPCR) followed by conventional PCR and Sanger sequencing were performed on serum identified with *E ewingii* as the sole disease-causing agent. Three months after diagnosis and treatment, no morulae were found, molecular testing for *E ewingii* detected no DNA, and convalescent IFA testing demonstrated a continued detection of antibodies for *E canis* at a 1:512 dilution. To the authors' knowledge, this is the first reported case of *E ewingii* confirmed with molecular diagnostics in a Texas dog. The zoonotic transmission potential of *E ewingii* should be noted as Texas supports competent tick vectors, and dogs represent effective sentinels for human ehrlichiosis. This report also highlights the utility of molecular diagnostics when serologic and microscopic evaluations are not sufficient in providing the species-level identity of a causative agent.

KEYWORDS
ehrlichiosis, morulae, polyarthritis, thrombocytopenia

1 | CASE PRESENTATION

An 8-year-old, neutered male, Golden Retriever presented for an apparent injury to the forelimb that was noted 4 days prior. Upon physical examination, there appeared to be joint effusion in both carpal joints. A CBC revealed leukopenia (4.49 K/μL; reference interval [RI] 5.50-16.90 K/μL) and thrombocytopenia (51 K/μL; RI 175-500 K/μL). No abnormalities were detected on an in-clinic serum chemistry panel (Glucose, BUN, Creatinine, BUN:Creatinine ratio, Phosphorus, Calcium, Total Protein, Albumin, Globulin, Albumin:Globulin ratio, ALT, ALKP, GGT, Total Bilirubin, Cholesterol, Sodium, Potassium, Sodium:Potassium ratio, Chloride). Samples were sent to the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) for blood smear review and serologic testing for tick-borne diseases. Wright's Giemsa stained blood smears revealed numerous morulae within neutrophils, suggestive of *Ehrlichia ewingii* or *Anaplasma phagocytophilum* (Figure 1). IgG antibodies against *Ehrlichia canis* were detected at a dilution of 1:512 via an indirect fluorescent antibody test (positive threshold of 1:64).[1]

Additional molecular testing was performed by purifying DNA from the serum sample as a more appropriate whole blood sample was not available. The patent-pending TickPath Layerplex qPCR assay (https://tvmdl.tamu.edu/tests/tickpath-layerplex-qpcr/), which simultaneously targets *E canis*, *E chaffeensis*, *E ewingii*, and *A phagocytophilum* DNA, detected *E ewingii* DNA only, revealing the differential diagnosis of ehrlichiosis. To verify the qPCR layerplex results in conjunction with results obtained by microscopic examination, an established conventional PCR protocol that targets the *16S rRNA* gene of *Ehrlichia* spp. and *Anaplasma* spp. was used.[2] However, initial analysis using the thermoprofile associated with the ECC and ECB primers detailed in the protocol proved insufficient in amplifying the positive suspect sample, along with a separate *E ewingii* control used for the PCR. Modifications to the thermoprofile, including increasing the annealing temperature and decreasing the time of extension, mitigated the amplification issue. The modified thermoprofile was as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension of 72°C for 7 minutes. The amplified product was then cleaned using the Wizard SV Gel and PCR Clean-Up Systems (Promega, Madison, WI, USA) followed by Sanger sequencing (Genewiz, Plainfield, NJ, USA). The obtained 444 base pair sequence (GenBank accession number MF893272) revealed 100% identity with the type strain sequence of *E ewingii* Stillwater (GenBank accession number NR_044747). Molecular examination by conventional PCR analysis also indicated the sample lacked DNA for all other closely related species (*E canis*, *E chaffeensis*, *A phagocytophilum*, and *A platys*).[2]

A tick-borne disease was suspected given the thrombocytopenia, joint effusion, and geographic location. The dog was prophylactically treated with Doxycycline (100 mg by mouth twice daily) until clinical signs resolved. Three months after diagnosis and treatment, no morulae were found on the blood smear examination, and DNA for *E ewingii* and closely related species (*E canis*, *E chaffeensis*, *A phagocytophilum*, and *A platys*), was not detected by qPCR on DNA extracted from a new whole blood sample. Convalescent indirect fluorescent antibody testing demonstrated a continued detection of antibodies for *E canis* at a dilution of 1:512. As of four months post treatment, the dog had fully recovered with no evidence of joint pain or effusion.

2 | DISCUSSION

Ehrlichiosis in dogs is caused by three species within the genus *Ehrlichia: E canis*, *E chaffeensis*, and *E ewingii*; and can present with a variety of clinical signs, such as lethargy, anorexia, evidence of bleeding, and polyarthritis.[3] While treatment is similar, clinical pathology findings can vary dramatically between dogs with ehrlichiosis; though, thrombocytopenia and mild anemia are common findings, but not definitive.[3,4] *E ewingii* infections in dogs are more likely to be associated with polyarthritis than other species of *Ehrlichia*.[3] Therefore, *E ewingii* should be considered in the differential diagnosis of dogs suspected of having polyarthropathy.

Like *E chaffeensis*, *E ewingii* is generally transmitted by the lone star tick (*Amblyomma americanum*), which currently remains the only proven competent vector.[4,5] Recently, the tick species *A inornatum* has also been identified as infected with *E ewingii*, though further studies would need to be conducted to definitively prove the vector competency of the tick species. In Texas, both species of opportunistic feeding ticks have been identified harboring *E ewingii*.[6,7] It has been accepted that *E ewingii* is a zoonotic pathogen initially recognized as a distinct etiologic agent of dogs in 1992 and later of humans in 1999. The Centers for Disease Control and Prevention began monitoring *E ewingii* infections in humans in 2008; however,

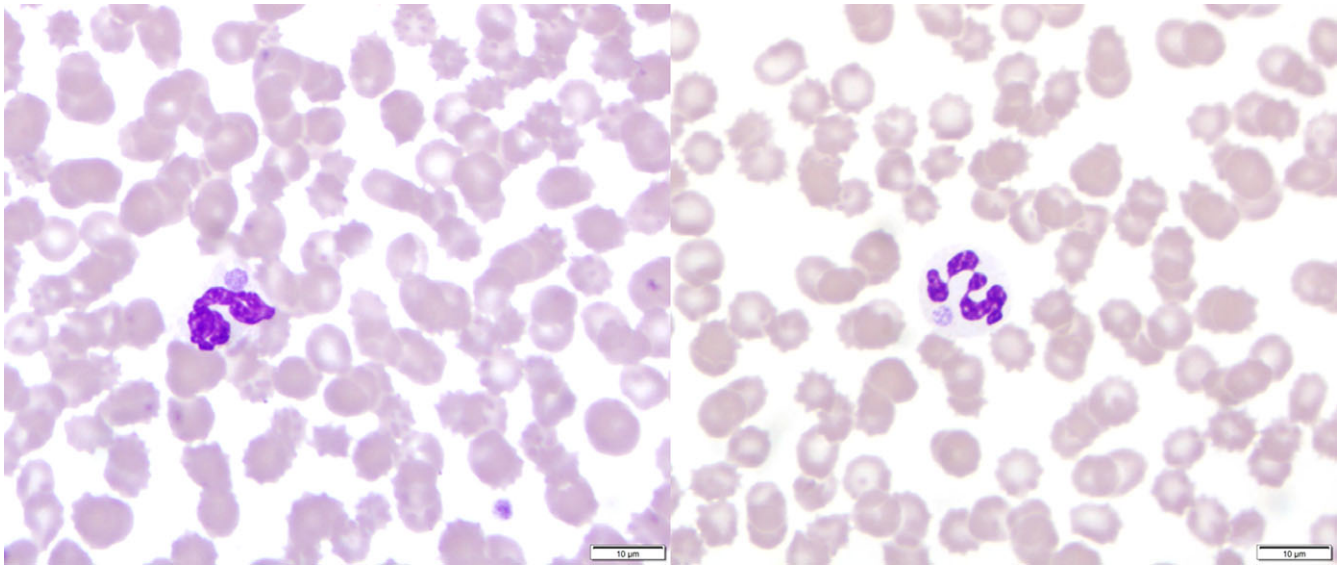

FIGURE 1 Peripheral blood smear from an 8-year-old castrated Golden Retriever. Neutrophils regularly contain intracytoplasmic morulae, later confirmed as *Ehrlichia ewingii* via PCR. The platelet mass is also markedly decreased. Modified Wright's Giemsa stain, 100× objective

the majority of ehrlichiosis cases are reported as *E chaffeensis* despite the potential that a proportion of cases currently reported might be miscategorized.[8] Furthermore, a recent study indicated *E ewingii* infections could be underreported in humans in the United States by as much as 7.0% due to limited molecular testing, unspecific serological tests, and disease treatment prior to etiologic diagnosis.[8]

In Texas, ehrlichiosis is primarily associated with *E chaffeensis* in humans and *E canis* in dogs with seroprevalence rates of 3.8% and 2.0%, respectively.[8,9] Although *E ewingii* has not been detected in Texas in recent reviews of human ehrlichiosis prevalence, this organism has been reported in Texas dogs, which serve as effective sentinels.[5,8,9] Further, all three *Ehrlichia* spp have an established serologic and molecular presence in dogs as shown in prevalence studies of states neighboring Texas, such as Oklahoma and Arkansas.[9,10] A concern remains, however, regarding the specificity of detection using serologic assays between other species of *Ehrlichia*, which may lead to pathogen misclassification.[8] Therefore, there is a need for the continued implementation of species-specific serologic tools when conducting *Ehrlichia* species seroprevalence studies on canines.[11]

Difficulties in diagnosing specific *Ehrlichia* species are due in part to the limitations of current screening methods. The popular IDEXX SNAP 4Dx Plus test used by veterinarians advertises detection of antibodies for *E canis* in addition to other unrelated vector-borne infections (*Dirofilaria immitis*, *Borrelia burgdorferi*, and *Anaplasma* spp.). However, as stated in a peer-reviewed IDEXX publication, this test will also detect antibodies recognizing *E ewingii* and *E chaffeensis* antigens; thus, true *Ehrlichia* species identification through this system may not be achievable.[12] Additionally, in serologic techniques used by diagnostic laboratories, antibodies against *E ewingii* may cross-react with targets intended for *E canis*, *E chaffeensis*, and *A phagocytophilum*, thus leading to further misclassifications of ehrlichiosis.[1,8]

While the morulae formed by *E canis* and *E chaffeensis* are generally found within monocytes, those formed by *E ewingii* are most commonly observed within neutrophils. In addition, the morulae of *E ewingii* appear identical morphologically to *A phagocytophilum*, thus confounding the microscopic discrimination between the two agents. However, molecular analysis through PCR techniques is considered the most sensitive and specific method to identify *Ehrlichia* species.[5] To the authors' knowledge, to date, *E ewingii* has not been identified using molecular techniques in Texas dogs.

To determine the species responsible for the morulae seen in this case, DNA was extracted from the serum sample and analyzed by layerplex qPCR methodology. qPCR analysis revealed the sample contained DNA for *E ewingii* but did not detect DNA for *E chaffeensis*, *E canis*, and *A phagocytophilum*. Subsequent analysis by conventional PCR corroborated the layerplex qPCR findings and allowed sequence analysis to definitively confirm an *E ewingii* infection.[2]

In summary, the diagnosis of ehrlichiosis is confounded by overlapping clinical presentations and nonspecific diagnostic testing modalities. Infections with *E ewingii* are likely underreported or unrecognized due to the known cross-reaction of anti-*Ehrlichia* sp. antibodies in serological tests. In addition, common molecular methodologies may require modifications to detect *E ewingii*, as detailed in this study. Furthermore, this case details the first molecular identification of *E ewingii* in a Texas dog. As Texas supports competent tick vectors for *E ewingii* and dogs represent effective sentinels for human ehrlichiosis, the increased zoonotic transmission potential of the disease should be considered. Therefore, both human and animal practitioners should consider this agent of ehrlichiosis when contemplating differential diagnoses.

DISCLOSURE

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

ORCID

Joseph J. Modarelli https://orcid.org/0000-0002-5543-7133
Maria D. Esteve-Gasent http://orcid.org/0000-0001-5921-2794

REFERENCES

1. Rodgers SJ, Morton RJ, Baldwin CA. A serological survey of *Ehrlichia canis*, *Ehrlichia equi*, *Rickettsia rickettsii*, and *Borrelia burgdorferi* in dogs in Oklahoma. *J Vet Diagn Invest*. 1989;1:154-159.
2. Wen B, Rikihisa Y, Mott JM, et al. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. *J Clin Microbiol*. 1997;35:1852-1855.
3. Neer TM, Breitschwerdt EB, Greene RT, Lappin MR. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. American College of Veterinary Internal Medicine. *J Vet Intern Med*. 2002;16:309-315.
4. Gieg J, Rikihisa Y, Wellman M. Diagnosis of *Ehrlichia ewingii* infection by PCR in a puppy from Ohio. *Vet Clin Pathol*. 2009;38:406-410.
5. Heitman K, et al. Increasing incidence of Ehrlichiosis in the United States: a summary of national surveillance of *Ehrlichia chaffeensis* and *Ehrlichia ewingii* infections in the United States, 2008–2012. *Am J Trop Med Hyg*. 2016;94:52-60.
6. Long SW, Pound JM, Yu XJ. *Ehrlichia* prevalence in *Amblyomma americanum*, Central Texas. *Emerg Infect Dis*. 2004;10:1342-1343.
7. Medlin JS, Cohen JI, Beck DL. Vector potential and population dynamics for *Amblyomma inornatum*. *Ticks Tick Borne Dis*. 2015;6:463-472.
8. Harris RM, Couturier BA, Sample SC, Coulter KS, Casey KK, Schlaberg R. Expanded geographic distribution and clinical characteristics of *Ehrlichia ewingii* infections, United States. *Emerg Infect Dis*. 2016;22:862-865.
9. Beall MJ, Alleman AR, Breitschwerdt EB, et al. Seroprevalence of *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in dogs in North America. *Parasit Vectors*. 2012;5:29.
10. Little SE, O'Connor TP, Hempstead J, et al. *Ehrlichia ewingii* infection and exposure rates in dogs from the southcentral United States. *Vet Parasitol*. 2010;172:355-360.
11. Qurollo BA, Chandrashekar R, Hegarty BC, et al. A serological survey of tick-borne pathogens in dogs in North America and the Caribbean as assessed by *Anaplasma phagocytophilum*, *A. platys*, *Ehrlichia canis*,

E. chaffeensis, *E. ewingii*, and *Borrelia burgdorferi* species-specific peptides. *Infect Ecol Epidemiol*. 2014;4.

12. IDEXX. The SNAP4Dx Plus Test provides sensitive and specific detection of tick-borne diseases, 2016; https://idexxcom-live-b02da1e51e754c9cb292133b-9c56c33.aldryn-media.com/filer_public/34/32/34328f81-21ee-47aa-bd34-29084292e22d/snap-4dx-plus-sensitivity-specificity.pdf. Accessed March 8, 2018.

How to cite this article: Modarelli JJ, Borst MM, Piccione J, Esteve-Gasent M. Molecular identification of *Ehrlichia ewingii* in a polyarthritic Texas dog. *Vet Clin Pathol*. 2019;48:96–99. https://doi.org/10.1111/vcp.12690