Novel *Rickettsia* Species Infecting Dogs, United States

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In 2018 and 2019, spotted fever was suspected in 3 dogs in 3 US states. The dogs had fever and hematological abnormalities; blood samples were *Rickettsia* seroreactive. Identical *Rickettsia* DNA sequences were amplified from the samples. Multilocus phylogenetic analysis showed the dogs were infected with a novel *Rickettsia* species related to human *Rickettsia* pathogens.

In the United States, tickborne *Rickettsia parkeri*, *R. philipii* (*Rickettsia* 364D), and *R. rickettsii*, causative agents of Rocky Mountain spotted fever (RMSF), are well-documented human spotted fever group (SFG) rickettsioses (*1*). *R. rickettsii* is the only known cause of SFG rickettsioses in dogs (*2*). The extent to which other SFG *Rickettsia* are pathogenic in dogs is unclear; however, SFG *Rickettsia* seroprevalence is high among dogs in the United States and Mexico (*3*,*4*). The increased *R. rickettsii* seroprevalence in humans in the United States during the past decade has been attributed to SFG *Rickettsia* cross-reactivity (*1*,*5*).

We report 3 dogs with febrile illness located in different US states. Samples from the dogs were *R. rickettsii* seroreactive. Identical *Rickettsia* DNA gene sequences were obtained from each dog's blood specimen and used to investigate *Rickettsia* spp.

The Cases

On May 15, 2018, a 10-year-old male neutered mixed breed dog (case 1) from Tennessee was examined by a veterinarian for lethargy and hyporexia. The owner reported removing a tick (species unknown) within the previous 2 weeks. On physical examination, the dog had fever (39.8°C) and possible hepatomegaly. Radiographic imaging results were unremarkable. Thrombocytopenia was the only abnormality noted on complete blood count (CBC). Serum biochemistry panel (SBP) abnormalities included hyperglobulinemia, increased serum alkaline phosphatase activity, hypoglycemia, and hyponatremia (Table 1). Results of urine dipstick and sediment examination were unremarkable. The dog's samples were *R. rickettsii* seroreactive and PCR positive for *Rickettsia* (Table 2). Clinical abnormalities resolved after treatment with doxycycline, and the dog remained healthy during the 1-year follow-up period.

On May 8, 2019, a 9-year-old male neutered Boston terrier (case 2) from Illinois was examined by a veterinarian for lethargy, difficulty walking, and painful elbows. Clinical signs developed 3 days after returning from a tick-infested area in Arkansas. Abnormalities noted on physical examination included fever (40.1°C), dehydration, joint effusion, elbow pain, and shifting leg lameness. Thrombocytopenia and mild leukocytosis were the only CBC abnormalities (Table 1). SBP abnormalities included hypoalbuminemia, increased alanine amino transferase activity, alkaline phosphatase activity, hypercholesterolemia, and hypocalcemia (Table 1). Mild microalbuminuria was noted. Neutrophilic inflammation was documented by synovial fluid cytology in the right and left stifle joints, right tarsus, and left elbow joint. The left carpus contained moderate, chronic inflammation with very rare extracellular cocci; however, culture resulted in no bacterial growth. The dog experienced cardiorespiratory arrest during sedated arthrocentesis but recovered after CPR and sedative reversal. Thoracic radiographs were unremarkable. The dog's samples were *R. rickettsii* seroreactive and PCR–positive for *Rickettsia* and convalescent titers demonstrated 4-fold seroconversion (Table 2). Most clinical abnormalities resolved after administration of doxycycline to treat rickettsiosis, prednisone to treat potential immune-mediated component, omeprazole to prevent gastric ulcers, and metronidazole to treat

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Table 1. Findings from physical examination, laboratory results, treatment regimens for 3 dogs infected with a novel *Rickettsia* species, United States*

Examination and treatment	Case 1	Case 2	Case 3
Physical examination	Febrile (39.8°C); lethargy; +/– hepatomegaly	Febrile (40.1°C); lethargy; dehydration; joint effusion (elbow, carpus, and tarsus); arthropathy; shifting leg lameness	Febrile (39.8°C); lethargy; abdominal pain
CBC	Platelets 141 × 10^3 cells/µL (RI 200–500 × 10^3 cells/µL)	Platelets 139 × 10^3 cells/µL (RI 170–400 × 10^3 cells/µL)	Platelets 60 × 10^3 cells/µL (RI 125–500 × 10^3 cells/µL); Hct 35.2% (RI 36%–55%); 2 d later platelets 25 × 10^3 cells/µL and Hct 26.8%
SBP	Globulins 4.5 g/dL (RI 2.1–4.4 g/dL); ALP 177 U/L (RI 11–140 U/L); glucose 73 mg/dL (RI 75–125 mg/dL); sodium 136.5 mmol/L (RI 143–153 mmol/L)	Albumin 2.2 g/dL (RI 2.7–4.4 g/dL); ALT 1,158 U/L (RI 12–118 U/L); ALP 1,702 U/L (RI 5–131 U/L); cholesterol 352 mg/dL (RI 92–324 mg/dL); calcium 8.4 mg/dL (RI 8.9–11.4 mg/dL)	Albumin 1.0 mg/dL (RI 2.5–4.3 mg/dL); calcium 8.4 mg/dL (RI 8.9–11.4 mg/dL); BUN 35 (35, RI 7–28 mg/dL)
Urinalysis	USG 1.007	Microalbuminuria 3.1 (RI <2.5 mg/dL)	USG 1.033; 3+ proteinuria; UPC 14.7 (RI 0.00–1.00)
Treatment regimen	Doxycycline (6 mg/kg every 12 h for 21 d)	Doxycycline (7 mg/kg every 12h for 28 d); prednisone (1 mg/kg every 12 h for 9 mo with gradual taper); omeprazole (1.4 mg/kg every 12h for 9 mo); ondansetron (0.5 mg/kg every 12h for 15 d); and metronidazole (17 mg/kg every 12 h for 15 d)	Doxycycline (7.5 mg/kg q12 h for 40 d); prednisone (1 mg/kg every 12 h for 14 d, then 0.5mg/kg every 12 h for 6 d, then every 24 h for 22 d until death), mycophenolate (12.5 mg/kg every 12 h for 22 d until death)

*ALP, alkaline phosphatase activity; ALT, alanine amino transferase activity; BUN, blood urea nitrogen; CBC, complete blood count; Hct, hematocrit; RI, reference interval; SBP, serum biochemistry panel; UA, urinalysis; UPC, urine protein/creatinine ratio; USG, urine specific gravity.

assumed dysbiosis. All SBP changes resolved within 5 months of treatment and the dog remained healthy during the 5-month follow-up.

On August 28, 2019, a 9-year-old male neutered terrier mixed-breed (case 3) from Oklahoma was examined by a veterinarian for lethargy, hyporexia, and polydipsia. Physical examination revealed fever (39.8°C) and palpable abdominal tenderness. CBC abnormalities included a normocytic normochromic anemia and thrombocytopenia. SBP abnormalities included hypoproteinemia, hypocalcemia, and mild azotemia. A protein-losing nephropathy (PLN) was documented by urine dipstick and protein/creatinine ratio. Blood samples were *R. rickettsii* seroreactive and *Rickettsia* PCR positive, and convalescent titers demonstrated 4-fold seroconversion (Table 2).

Table 2. CVBD diagnostic results for blood and serum samples from 3 dogs infected with a novel *Rickettsia* species*

			Rickettsia PCR§								
						gltA region			*ompA* region		
Sample dates	CVBD panel†	IFA‡	23S-5S ITS	*htrA* (17kDa)	*mmpA-purC* ITS	1	2	3	1	2	3
Case 1											
2018 May 5¶	–	1:512	+	+	+	+	+	+	+	+	–**
Case 2											
2019 May 8¶	–	1:256	+	+	+	+	NA††	+	+	+	NA††
2019 May 15	–	1:8,192	–	NA	NA	NA	NA	NA	NA	NA	NA
2019 May 28	–	1:1,024	–	NA	NA	NA	NA	NA	NA	NA	NA
2019 Jul 16	NA	1:2,048	NA	NA	NA	NA	NA	NA	NA	NA	NA
2019 Oct 2	–	1:2,048	–	NA	NA	NA	NA	NA	NA	NA	NA
2019 Nov 12	–	1:2,048	–	NA	NA	NA	NA	NA	NA	NA	NA
Case 3											
2019 Aug 28¶‡‡	–	1:1,024	+	+	+	+	+	+	+	+	+
2019 Sep 10	NA	1:8,192	–	NA	NA	NA	NA	NA	NA	NA	NA

*CVBD, canine vectorborne disease; IFA, immunofluorescence assay; NA, not applicable; +, positive; –, negative.
†Panel includes IFA serology for *Babesia canis vogeli*, *B. gibsoni*, *Bartonella henselae*, *B. koehlerae*, *B. vinsonii berkhoffii*, and *Ehrlichia canis*; point-of-care ELISA serology test SNAP 4DX Plus for *Dirofilaria immitis* antigen and antibodies against *Anaplasma phagocytophilum*, *A. platys*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *E. ewingii*; and PCR for *Anaplasma*, *Babesia*, *Bartonella*, *Ehrlichia*, hemotropic *Mycoplasma, Neoehrlichia*, and *Neorickettsia*.
‡IFA results are reported as reciprocal titers. All samples were positive for *R. rickettsii.*
§PCR assay gene targets 23S-5S ITS, *htrA* (17 kDa), *mmpA-purC* ITS, *gltA*, and *ompA*.
¶Sample tested before doxycycline treatment administered.
**The PCR was negative despite repeated attempts. *ompA* region 3 PCR assay was designed to bridge *ompA* regions 1 and 2 to obtain an additional 281 bps. The total amplicon size of *ompA* region 3 is 533 bp (Appendix Table, https://wwwnc.cdc.gov/EID/article/26/12/20-0272-App1.pdf). DNA from case 1 was ≥1 year old when retrospective PCRs were performed. Poor DNA quality might have prevented amplification of the larger amplicon.
††PCR assays were not performed due to depleted blood sample for DNA extraction and testing.
‡‡GenBank accession nos. for sequences from case 3: 23S-5S ITS, MT050448; *htrA* (17 kDa), MT050446; *mmpA-purC* ITS, MT066187; *gltA*, MT050445; and *ompA*, MT050447.

The year before, in August 2018, the dog described in case 3 was examined by a veterinarian for lethargy after tick attachment. At that time, fever (39.7°C), anemia, thrombocytopenia, hyperbilirubinemia, and hypoproteinemia were documented. IFA serology tests performed by 1 diagnostic laboratory showed samples were *R. rickettsii* seroreactive (1:320) but seronegative for *Anplasma* spp., *Borrelia burdorferi*, and *Ehrlichia* spp. by SNAP 4Dx Plus (IDEXX Laboratories, https://www.idexx.com). Doxycycline and immunosuppressive doses of prednisone were administered concurrently for RMSF and potential immune-mediated disease. Clinical and hematologic abnormalities resolved, and treatment was transitioned from prednisone to cyclosporine due to adverse side effects. Cyclosporine was discontinued in January 2019 and serial monthly CBCs remained normal through March 2019. When rechecked on August 9, 2019, for joint pain, hematocrit and platelet count were normal, but hypoproteinemia, hypoalbuminemia, and hypocalcemia were detected. By August 30, 2019, the dog's anemia and thrombocytopenia worsened, despite treatment with doxycycline and prednisone. Marked abdominal effusion was documented by abdominal ultrasound, without evidence of an intra-abdominal mass. Prednisone and mycophenolate were administered for presumptive immune-mediated thrombocytopenia, and within 3 weeks, the platelet count normalized and titers increased by 4-fold. Despite medical therapy for PLN, nephrotic syndrome developed, and the dog was euthanized.

We obtained identical *Rickettsia* DNA gene sequences from each dog's blood specimen. We confirmed novel *Rickettsia* sp. by PCR targeting 3 genes (*gltA*, *htrA*, and *ompA*) and 2 intergenic spacer re-

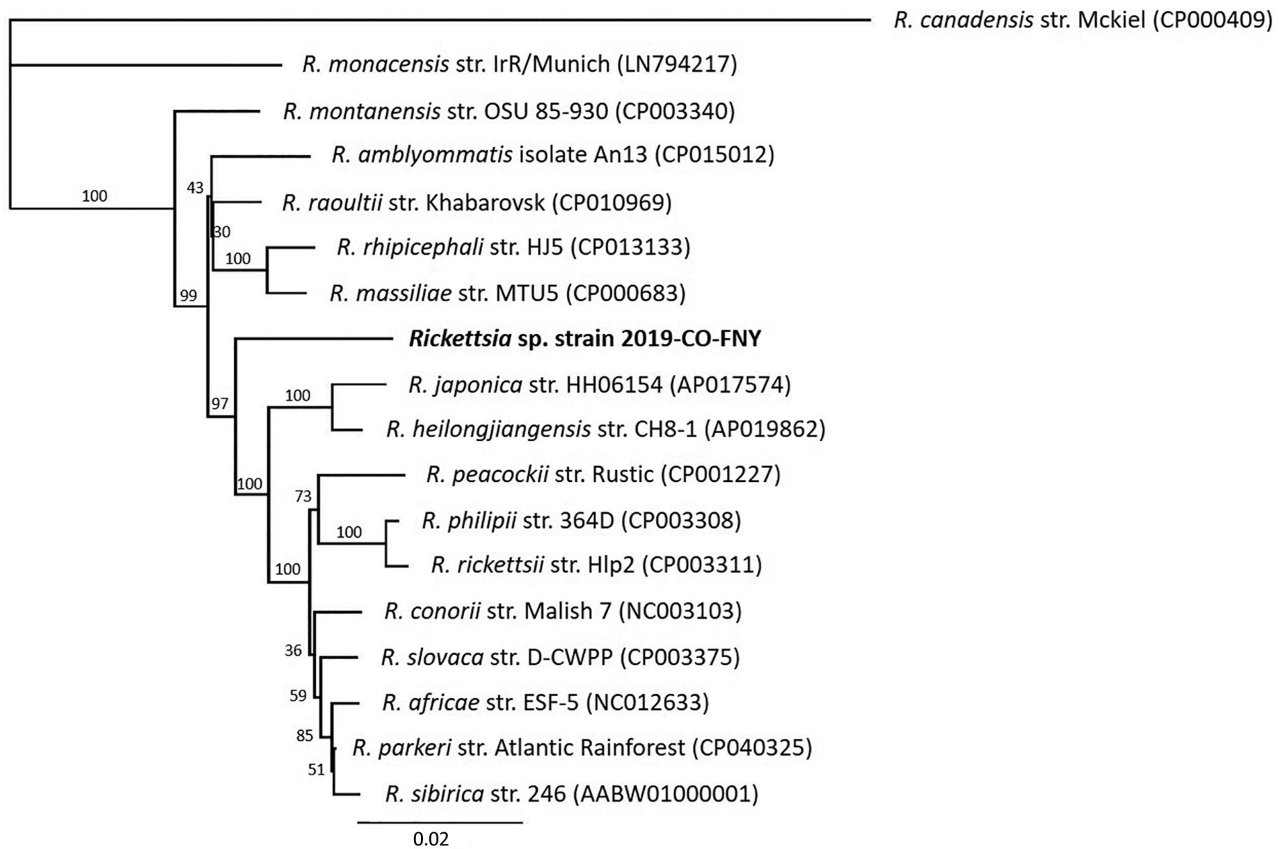

Figure. Multilocus phylogenetic tree of *Rickettsia* spp. obtained from a dog with Rocky Mountain spotted fever–type symptoms in 2019 (bold) compared with reference sequences. We noted 3 dogs with RSMF symptoms. *Rickettsia* DNA were identical among all 3 cases; however, complete sequences from all 5 regions were obtained only from case 3, which we used to represent the novel *Rickettsia* species strain 2019-CO-FNY. We used 2,576 nucleotides concatenated from regions within 3 genes (*gltA*, *htrA*, and *ompA*) and 2 intergenic spacer regions (23S-5S and *mmpA-purC*). We used the maximum-likelihood method and Tamura-Nei model (*6*,*7*) optimized for branch length, topology, and substitution rate to assemble the tree by using the PhyML 3.3.20180621 plugin in Geneious Prime 11.0.0+7 (https://www.geneious.com). Numbers at nodes indicate bootstrap percentages obtained from 1,000 resamplings. Numbers in parentheses are GenBank accession numbers. The tree is drawn to scale. Scale bar indicated the number of nucleotide substitutions per site.

gions (23S-5S and *mmpA-purC*) (Table 2). *Rickettsia* amplicons were 100% identical among the 3 dogs. We amplified a larger region of the *ompA* and *gltA* genes by using 3 different quantitative PCRs from case 3. We submitted sequences from this dog's serum samples to GenBank (accession nos. MT050445–8 and MT066187). We also used the *Rickettsia* sequences from case 3 to generate a phylogenetic tree (Table 2) based on concatenated novel *Rickettsia* sp. DNA sequences and reference *Rickettsia* spp. We generated the phylogenetic tree by using the maximum-likelihood method based on the Tamura-Nei model (Figure) (*6*,*7*). Multilocus phylogenetic analysis placed the novel *Rickettsia* sp. in a clade among SFG *Rickettsia* between the human pathogens *R. heilongjiangensis* and *R. massiliae*. We attempted cell culture isolation of the *Rickettsia* sp. from whole blood but were unsuccessful (Appendix, https://wwwnc.cdc.gov/EID/article/26/12/20-0272-App1.pdf).

Conclusions

We report similar illnesses among 3 dogs from different US states associated with tick exposures occurring in summer months. All 3 cases demonstrated fever, lethargy, and thrombocytopenia, abnormalities commonly associated with RMSF. Case 1 had a typical acute onset fever and rapidly responded to treatment with doxycycline; case 2 had a neutrophilic polyarthritis, which has been associated with RMSF in dogs (*8*). Case 3 was examined for acute onset febrile illness 1 year before the novel *Rickettsia* sp. infection was documented; *Rickettsia* IFA seroreactivity was documented on both occasions. This dog likely had an unidentified, concurrent disease process that contributed to PLN.

The cases were geographically distributed among 4 states; the dogs resided in Illinois, Oklahoma, and Tennessee, but the dog from Illinois had traveled to a tick-infested area of Arkansas. The tick species were not identified, but ticks common to these states include *Amblyomma americanum, Dermacentor variabilis,* and *Rhipicephalus sanguineus* sensu lato, all of which are known to transmit *Rickettsia* (*3*). *Haemophysalis longicornis*, an invasive tick species recently confirmed in the United States, including in Tennessee and Arkansas, should be considered a potential vector for *Rickettsia* spp. (*9*,*10*).

Based on serologic cross-reactivity, presence of *ompA*, and phylogenetic tree analysis, the new *Rickettsia* sp. is an SFG *Rickettsia*, phylogenetically related to human pathogenic *R. heilongjiangensis* and *R. massiliae*, with only 95% identity to each (*11*,*12*). Thus, we report a previously unknown and unique *Rickettsia* sp. with clinical significance for dogs and potentially humans. Because this novel *Rickettsia* cross-reacts with *R. rickettsia* on IFA, it could be underdiagnosed and more geographically widespread. Studies aimed at identifying the tick vector, potential animal reservoirs, and prevalence are ongoing. These 3 canine rickettsioses cases underscore the value of dogs as sentinels for emerging tickborne pathogens (*13*,*14*).

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Mr. Wilson is a vectorborne disease research technician at the North Carolina State Vector Borne Disease Diagnostic Lab. His research interests include optimization of molecular testing and vectorborne infectious disease diagnoses.

References

1. Straily A, Stuck S, Singleton J, Brennan S, Marcum S, Condit M, et al. Antibody titers reactive with *Rickettsia rickettsii* in blood donors and implications for surveillance of spotted fever Rickettsiosis in the United States. J Infect Dis. 2020;221:1371–8. https://doi.org/10.1093/infdis/jiz316
2. Warner RD, Marsh WW. Rocky Mountain spotted fever. J Am Vet Med Assoc. 2002;221:1413–7. https://doi.org/10.2460/javma.2002.221.1413
3. Hardstone Yoshimizu M, Billeter SA. Suspected and confirmed vector-borne rickettsioses of North America associated with human diseases. Trop Med Infect Dis. 2018;3:2. https://doi.org/10.3390/tropicalmed3010002
4. Yancey CB, Hegarty BC, Qurollo BA, Levy MG, Birkenheuer AJ, Weber DJ, et al. Regional seroreactivity and vector-borne disease co-exposures in dogs in the United States from 2004–2010: utility of canine surveillance. Vector Borne Zoonotic Dis. 2014;14:724–32. https://doi.org/10.1089/vbz.2014.1592
5. Drexler NA, Dahlgren FS, Heitman KN, Massung RF, Paddock CD, Behravesh CB. National surveillance of spotted fever group rickettsioses in the United States, 2008–2012. Am J Trop Med Hyg. 2016;94:26–34. https://doi.org/10.4269/ajtmh.15-0472
6. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10:512–26. https://doi.org/10.1093/oxfordjournals.molbev.a040023
7. Guindon S, Dufayard JF, Lefort V, Anisimova M,

Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010;59:307–21. https://doi.org/10.1093/sysbio/syq010
8. Gasser AM, Birkenheuer AJ, Breitschwerdt EB. Canine Rocky Mountain spotted fever: a retrospective study of 30 cases. J Am Anim Hosp Assoc. 2001;37:41–8. https://doi.org/10.5326/15473317-37-1-41
9. Raghavan RK, Barker SC, Cobos ME, Barker D, Teo EJM, Foley DH, et al. Potential spatial distribution of the newly introduced long-horned tick, *Haemaphysalis longicornis* in North America. Sci Rep. 2019;9:498. https://doi.org/10.1038/s41598-018-37205-2
10. Xu H, Zhang Q, Guan H, Zhong Y, Jiang F, Chen Z, et al. High incidence of a novel *Rickettsia* genotype in parasitic *Haemaphysalis longicornis* from China-North Korea Border. Sci Rep. 2019;9:5373. https://doi.org/10.1038/s41598-019-41879-7
11. Ando S, Kurosawa M, Sakata A, Fujita H, Sakai K, Sekine M, et al. Human *Rickettsia heilongjiangensis* infection, Japan. Emerg Infect Dis. 2010;16:1306–8. https://doi.org/10.3201/eid1608.100049
12. Vitale G, Mansuelo S, Rolain J-M, Raoult D. *Rickettsia massiliae* human isolation. Emerg Infect Dis. 2006;12:174–5. https://doi.org/10.3201/eid1201.050850
13. Estrada I, Balagot C, Fierro M, Kriner P, Iniguez-Stevens E, Kjemtrup A, et al. Spotted fever group *rickettsiae* canine serosurveillance near the US-Mexico border in California. Zoonoses Public Health. 2020;67:148–55. https://doi.org/10.1111/zph.12666
14. Smith FD, Ballantyne R, Morgan ER, Wall R. Estimating Lyme disease risk using pet dogs as sentinels. Comp Immunol Microbiol Infect Dis. 2012;35:163–7. https://doi.org/10.1016/j.cimid.2011.12.009

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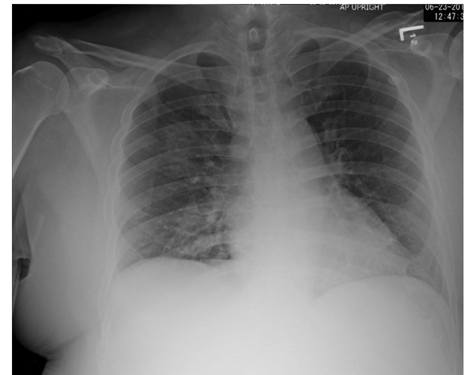

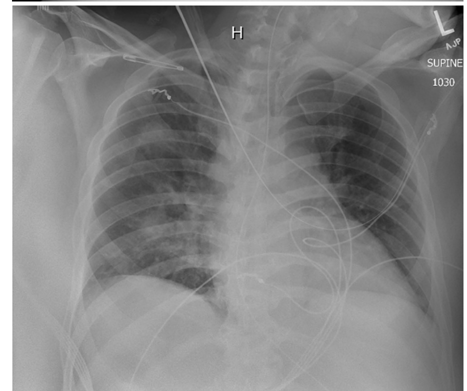

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Appendix

Molecular Diagnostic Testing

EDTA-anticoagulated whole blood was submitted to the North Carolina State College of Veterinary Medicine, Vector-Borne Disease Diagnostic Laboratory (VBDDL) for vector-borne disease PCR testing. DNA was extracted from 200 µL aliquots of whole blood by using a QIAsymphony SP robot (QIAGEN, https://www.qiagen.com) and QIAsymphony DNA Mini Kit (QIAGEN). All sets of extractions included negative extraction controls of molecular-grade water. DNA was stored at −20°C before PCR testing. The absence of PCR inhibitors was demonstrated by an internal control quantitative PCR (qPCR) designed to amplify the host glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*1*). Samples were tested for *Rickettsia* spp. DNA by using *Rickettsia*-specific PCR assays targeting 6 different DNA regions (Appendix Table). All PCR assays were run with negative molecular-grade water, a negative control of known uninfected canine DNA, and a DNA–positive control extracted from *R. conorii* and *R. rickettsia* cultures. Amplification assays were performed in CFX96 Real-Time Detection System combined with C1000 Thermal Cycler (Bio-Rad, https://www.bio-rad.com) for qPCR and an Eppendorf Mastercycler EPgradient with aluminum block for cPCR. Amplification reactions contained 12.5 µL SYBR Green Supermix (Bio-Rad) for qPCRs or MyTaq HS Mix (2×) (Bioline, https://www.bioline.com) for cPCRs, 5 µL of DNA template, primers at final concentration of 0.4 µMol and molecular-grade water to a final volume of 25 µL. We provide details on PCR reaction conditions and *Rickettsia* gene targets and references for PCRs we did not develop (*2*–*6*) (Appendix Table).

Rickettsia rickettsii IFA Serologic Testing

Serial, 2-fold dilutions of canine serum samples were made in PBS solution containing 0.05% Tween 20, 0.5% non-fat dry milk, and 1% normal goat serum (GIBCO, Fisher Scientific, https://www.fishersci.com) before adding 8–10 µL to slide wells prepared with *R. rickettsia,* originally isolated from a naturally infected dog, cultured in DH82 cells. Slides containing serial diluted canine serum was incubated in a humidified chamber at 37°C for 30 minutes and washed in PBS at room temperature at 300 rpm for 30 minutes. Slides were then air-dried before adding 8–10 µL of a 0.01 mg/mL solution of fluorescein isothiocyanate (FITC) goat anti-dog immunoglobulin G (H&L) conjugate (Sigma, https://www.sigmaaldrich.com) to each well. Slides were then incubated in a humidified chamber at 37°C for 30 min before being washed in ≈400 mL PBS at room temperature, in the dark, at 300 rpm for 20 min. Slides were washed for an additional 20 min after adding 4–5 drops of a Tween-20. Slides were then rinsed with deionized water and dried in the dark before adding a coverslip with antifading mounting medium, Vectashield (Vector Laboratories, https://vectorlabs.com). Slides were evaluated by using a ZEISS Axio Lab.A1 fluorescence microscope with exciter and barrier filters (Carl Zeiss Microscopy, https://www.zeiss.com) under ×400 magnification. Each slide contained canine seroreactive *R. rickettsia* positive control serum and canine nonreactive negative control serum. Canine serum samples were screened at 1:16, 1:32 and 1:64 dilutions, and all serum samples reactive at a titer of 1:64 were repeated and diluted to an endpoint titer of 1:8,192. To avoid confusion with possible nonspecific binding found at low dilutions, a cutoff titer of ≥1:64 was used to define a seroreactive titer.

Rickettsia Culture

For cases 1 and 2, we attempted to grow the new *Rickettsia* sp. in DH82 canine cells with a modified protocol (*7*). EDTA-anticoagulated whole blood (0.5 mL) was added to a 95% confluent monolayer of DH82 canine cells in a T-10 flask with 1 mL of media (RPMI/10% FBS). The flask was rocked slowly at room temperature for 1 hour before adding 3 mL of fresh media (RPMI/10% FBS) and placing in a 37°C incubator with 5% CO_2. After 12 hours of incubation, all nonadherent content was added to a T-25 flask of 85% confluent DH82 cells. Fresh media was added to both flasks and placed in a 37°C incubator with 5% CO_2. After 12

hours of incubation, the contents of both T-10 and T-25 flasks were combined into a T-75 flask, and fresh media was added to the used T-10 and T-25 flasks. Content from all flasks were incubated for 2 weeks and periodically tested by *Rickettsia* qPCR and Diff-Quick staining (RAL Diagnostics, https://www.ral-diagnostics.fr) to identify *Rickettsia* organisms.

Sequencing and Phylogenetic Analyses

Nucleotide sequencing of amplicons was performed by Genewiz, Inc. (https://www.genewiz.com) by using both forward and reverse primers for each DNA target. DNA sequences were analyzed by using the BLAST search algorithm and the NCBI nucleotide database and sequence alignments were performed by using Geneious Prime (https://www.geneious.com) for Java version 11.0.2+7. Sequences were deposited into NCBI nucleotide database. A total of 2,576 aligned nucleotides from regions within 3 genes (*ompA*, *gltA*, and 17 kDa) and 2 intergenic spacers (23S-5S and *mmpA-purC*), were concatenated and a multilocus phylogenetic tree was assembled by using maximum-likelihood method and Tamura-Nei model through PhyML 3.3.20180621and Geneious Prime software (*8*,*9*).

References

1. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J Clin Microbiol. 2003;41:4172–7. PubMed https://doi.org/10.1128/JCM.41.9.4172-4177.2003

2. Tyrrell JD, Qurollo BA, Tornquist SJ, Schlaich KG, Kelsey J, Chandrashekar R, et al. Molecular identification of vector-borne organisms in *Ehrlichia* seropositive Nicaraguan horses and first report of *Rickettsia felis* infection in the horse. Acta Trop. 2019;200:105170. PubMed https://doi.org/10.1016/j.actatropica.2019.105170

3. Kidd L, Maggi R, Diniz PP, Hegarty B, Tucker M, Breitschwerdt E. Evaluation of conventional and real-time PCR assays for detection and differentiation of spotted fever group rickettsia in dog blood. Vet Microbiol. 2008;129:294–303. PubMed https://doi.org/10.1016/j.vetmic.2007.11.035

4. Eremeeva ME, Dasch GA, Silverman DJ. Evaluation of a PCR assay for quantitation of *Rickettsia rickettsii* and closely related spotted fever group rickettsiae. J Clin Microbiol. 2003;41:5466–72. PubMed https://doi.org/10.1128/JCM.41.12.5466-5472.2003

5. Peckle M, Luz HR, Labruna MB, Serpa MCA, Lima S, Maturano R, et al. Multi-locus phylogenetic analysis groups the New World bacterium *Rickettsia* sp. strain ApPR with the Old World species *R. africae*; proposal of "*Candidatus* Rickettsia paranaensis". Ticks Tick Borne Dis. 2019;10:101261. PubMed https://doi.org/10.1016/j.ttbdis.2019.07.005

6. Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, et al. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil, where Brazilian spotted fever is endemic. J Clin Microbiol. 2004;42:90–8. PubMed https://doi.org/10.1128/JCM.42.1.90-98.2004

7. Luce-Fedrow A, Wright C, Gaff HD, Sonenshine DE, Hynes WL, Richards AL. In vitro propagation of *Candidatus* Rickettsia andeanae isolated from *Amblyomma maculatum.* FEMS Immunol Med Microbiol. 2012;64:74–81. PubMed https://doi.org/10.1111/j.1574-695X.2011.00905.x

8. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10:512–26. PubMed https://doi.org/10.1093/oxfordjournals.molbev.a040023

9. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010;59:307–21. PubMed https://doi.org/10.1093/sysbio/syq010

Appendix Table. Quantitative and conventional PCR assay conditions, PCR targets, primers, and associated references used in an investigation of novel *Rickettsia* in dogs, USA

Gene target (*reference*)	Primers	Primer Sequence (5′–3′)	Primer, μMol	PCR product, bp	Polymerase A/D	D	A	E	No. DAE cycles	Final E
23s-5s ITS (*2*)	Rick23-5_F2	AGC TCG ATT GAT TTA CTT TGC TG	0.4	215	98°C, 180 s	98°C, 15 s	62°C, 15 s	72°C, 20 s	40	NA
	Rick23-5_R	CCA CCA AGC TAG CAA TAC AAA	0.4							
OmpA_1† (*3*)	OmpA 107F	GCT TTA TTC ACC ACC TCA AC	0.4	137	95°C, 120 s	95°C, 10 s	58°C, 10 s	72°C, 10 s	50	72°C, 30 s
	OmpA 299R	TRA TCA CCA CCG TAA GTA AAT	0.4							
OmpA_2 (*4*)	Rr190.547F	CCT GCC GAT AAT TAT ACA GGT TTA	0.4	115	98°C, 180 s	98°C, 15 s	60°C, 15 s	72°C, 20 s	40	
	Rr190.701R	GTT CCG TTA ATG GCA GCAT	0.4							
OmpA_3 (*4*)	OmpA 107F	GCT TTA TTC ACC ACC TCA AC	0.4	533	95°C, 120 s	95°C, 10 s	60°C, 10 s	72°C, 10 s	50	72°C, 30 s
	Rr190.701R	GTT CCG TTA ATG GCA GCAT	0.4							
17kDa	Rck 17kDa F	GCG CAT TAC TTG GTT CTCA A	0.4	173	98°C, 180 s	98°C, 15 s	60°C, 15 s	72°C, 15 s	40	NA
	Rck 17kDa R	GTA GAA TGG CGT AAT CCG GA	0.4							
mmpA-purC ITS† (*5*)	mppA F	CAA ATG GCT CAA GAG AAA AA	0.4	507	95°C, 120 s	95°C, 15 s	60°C, 15 s	72°C, 30 s	40	72°C, 60 s
	mppA R	TTT TCA ATG CCG ATC ATT TC	0.4							
GltA_1†	GltA F	TGC GGA AGC CGA TTG CTT TAC	0.4	847	95°C, 120 s	95°C, 10 s	58°C, 10 s	72°C, 10 s	50	72°C, 30 s
	GltA R	AGC TGC CCG AGT TCC TTT AAT AC	0.4							
GltA_2	GltA F2	CAG TAC TTA AAG AAA CGT GCA AAG	0.4	222	98°C, 180 s	98°C, 15 s	57°C, 15 s	72°C, 20 s	40	NA
	CS-6	AGG GTC TTC GTG CAT TTC TT	0.4							
GltA_3 (*6*)	CS-5	GAG AGA AAA TTA TAT ATC CAA ATG TTG AT	0.4	99	98°C, 180 s	98°C, 15 s	57°C, 15 s	72°C, 20 s	40	NA
	CS-6	AGG GTC TTC GTG CAT TTC TT	0.4							

*A, annealing; A/D, activation/denaturization; D, denaturization; DAE, denaturation-annealing-extension; E, extension.
†Conventional PCR.