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Retrospective Clinical and Molecular Analysis of Conditioned Laboratory Dogs (*Canis familiaris*) with Serologic Reactions to *Ehrlichia canis*, *Borrelia burgdorferi*, and *Rickettsia rickettsii*

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Dogs are susceptible to different tickborne infections, including members of the *Anaplasmataceae* (*Ehrlichia canis*, *E. ewingii*, *E. chaffeensis*, *Anaplasma phagocytophilum*, *A. platys*), *Borrelia burgdorferi*, and *Rickettsia rickettsii*. These diseases can manifest with clinical signs including fever, anorexia, malaise, lameness, rash, and bleeding episodes; however, these signs are nonpathognomonic, and infections can occur in the absence of clinical signs. Hematologic abnormalities can include leukopenia, thrombocytopenia, hyperproteinemia and hypergammaglobulinemia. In biomedical research, diseases such as canine monocytic ehrlichiosis, Lyme disease, and Rocky Mountain spotted fever may cause morbidity among exposed dogs and confound research results. Random-source dogs are susceptible to these diseases because of their increased risk of arthropod exposure. Nonpurpose bred, randomly selected conditioned dogs (n = 21) were examined; blood samples were taken for hematology, biochemistry analysis, tickborne pathogen serology, and PCR. Of these, 2 dogs (10% of the population) presented with illness characterized by fever, malaise, lameness, or hemostatic abnormalities, and 15 (71%) had antibodies to one or more tickborne pathogens. No specific hematologic or biochemical differences were apparent between seronegative dogs and seropositive dogs reactive to all 3 pathogens. *E. canis* and *B. burgdorferi* PCR of tissues and blood were negative for all dogs. PCR amplification of several *Ehrlichia* and *Anaplasma* genes yielded no positive samples. From this cohort of dogs, serologic and molecular results indicate prior exposure without active infection or clinical disease. Exposure to and potential for infection with these bacteria and other pathogens may contribute to blood and tissue alterations that could confound experiments and lead to misinterpretation of data in canine models.

Abbreviations: CBC, complete blood count

Some research investigations continue to rely on conditioned, random-source hounds not obtained from research facilities providing animals, and thus, prior or concurrent disease conditions that could affect research results are encountered frequently. Reasons for continued use of Class B dogs in biomedical research include lower cost, flexibility in the size and age of animals, and appropriateness for acute, terminal procedures. In our program, we identified ectoparasite-borne diseases, such as heartworm disease transmitted by mosquitoes and tapeworm infestation acquired by ingestion of fleas, in random-source dogs. In addition, we recently identified increased tickborne pathogen exposure in random-source dogs, including 3 frequently reported canine tickborne pathogens: *Ehrlichia canis, Borrelia burgdorferi*, and *Rickettsia rickettsii*.[3,4,29] These 3 bacteria do not always cause overt clinical disease, but both *E. canis* and *B. burgdorferi* can persist and cause chronic immune stimulation[2,20,21,29,30,36] that could affect physiologic responses when various disease conditions are modeled.

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This retrospective investigation was initiated due to an index clinical case, in which a dog instrumented for a cardiac study was found depressed and lethargic with an undulating fever reaching 40.0°C. Physical examination was performed and provided no indication that the illness was experimentally induced. The examination revealed petechiation of the oral mucosa and shifting leg lameness, with minor swelling of the right elbow, which appeared to involve the subcutaneous tissues and not the elbow joint. Percutaneous cardiac instrumentation had been performed a week prior to illness, without evidence of wound infection that could explain the dog's condition. Cultures were taken around the catheter exit site and revealed growth of skin commensals such as *Staphylococcus* and *Streptococcus* that were sensitive to most antibiotics screened. Blood cultures were not conducted in light of recent use of perioperative antibiotics. A complete blood count (CBC) revealed a white blood cell count of 22.3×10^3 cells/μl (normal range, 4 to 15.5×10^3 cells/μl), which reached a high of 37.4×10^3 cells/μl 4 d later and began to resolve 1 wk after treatment with additional antibiotics and supportive care. Other than leukocytosis, the CBC values were within normal limits. Platelets were counted each time a CBC was performed and did reach levels that were below normal range (170 to 400×10^3 cells/μl), but at the time of initial clinical presentation, the platelet count (266×10^3 cells/μl) was within normal limits. Biochemistry analysis revealed mild hyperproteinemia of 7.5 g/dl (range, 5.0 to 7.4 g/dl) and hy-

perglobulinemia of 4.6 g/dl (range, 1.6 to 3.6 g/dl), and protein electrophoresis revealed hypergammaglobulinemia (polyclonal gammopathy). The differential diagnosis for hypergammaglobulinemia includes multiple myeloma, chronic inflammatory disease, hyperimmunization, acute infection, and chronic liver disease.[17] However, due to the likelihood of significant ectoparasite exposure in many random-bred dogs, a tickborne infectious etiology was suspected, although septicemia could not be ruled out definitively.

Serologic tests of this dog (02-283) for *E. canis, B. burgdorferi*, and *R. rickettsii* were performed and demonstrated elevated titers for all 3 pathogens (Table 1), although we were unable to confirm active infection. The dog was treated with 10 mg/kg doxycycline daily for 10 d, later supplemented with enrofloxacin; clinical signs and leukocytosis resolved substantially within a few days after treatment and completely within a week of treatment. Four weeks later, serology was repeated and revealed decreased titers for all 3 pathogens, specifically *E. canis* (160 to 40), *B. burgdorferi* (128 to negative), and *R. rickettsii* (256 to 128). These results prompted a retrospective serosurvey of tickborne bacterial titers among our random-source Class B dogs to better understand degree of pathogen exposure and potential correlation with active infection (by PCR) and clinical disease (physical examination and bloodwork). The hypothesis generated from this index case was that dogs were infected by these bacteria during prior tick bites, after which the dogs developed detectable antibody titers, suppressed active infection, and were either not infected or maintained a very low infection level that sustained the sequelae of immune stimulation at the time of study. Because the study was retrospective, some baseline and follow-up samples (blood, tissues, synovial fluid) were not collected or could not be retrieved.

Materials and Methods

Animals. Twenty-one conditioned dogs were acquired from a Class B United States Department of Agriculture vendor for use in canine cardiac studies during mid-2002 through early 2003. Dogs were large hound breeds; many were retired hunting dogs from diverse geographic locations, primarily central US states, although other geographic originations were possible. Dogs were preconditioned before arrival at our institution with antiparasitic prophylaxis, heartworm testing, and vaccinations for distemper, parvovirus, canine hepatitis, parainfluenza, coronavirus, *Bordetella*, and rabies. On arrival, all dogs were given a complete physical examination, body condition score, application of fipronil (Frontline, Merial, Duluth, GA), and single dose of praziquantel–pyrantel pamoate–febantel (Drontal Plus, Bayer, Pittsburgh, PA), and blood was taken for CBC, chemistry, and tick serology profile. No ectoparasites were noted on these study animals. Once in our animal facility, dogs were housed and given exercise in accordance with the Animal Welfare Act[1] and the *Guide for the Care and Use of Laboratory Animals*;[31] our program is fully accredited by AAALAC. Research studies involving these animals were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. All dogs used were placed under the same cardiac failure protocol, and instrumentation was conducted in all animals by either a closed (vascular access pacemaker placement only) or open (pacemaker with heart instrumentation via thoracotomy) approach. Dogs were euthanized at the conclusion of experimental studies when preset time points were reached or if the animal developed heart failure and weight loss too complex to manage clinically.

CBC and biochemistry analysis. Approximately 4 ml of whole blood was drawn for CBC and biochemistry analysis (Antech Diagnostics, Lake Success, NY) on arrival at the institution as part of normal preventative medicine and quarantine procedures. Dogs received baseline blood sampling on entry into experimental use, and blood sampling was repeated only if indicated for illness or experimental purposes.

Serology. Serology (Antech Diagnostics) was conducted as per protocol for Class B dogs and included 3 tickborne infectious agents as part of a standard tickborne agent screening panel. Serology results were interpreted per laboratory guidelines as follows: *Ehrlichia canis* (IgG), a titer of less than 20 was considered negative, whereas IgG greater than or equal to 20 was positive, with a positive titer supporting exposure to *E. canis* or crossreactive *Ehrlichia* spp.; *B. burgdorferi* (IgG), a titer of less than 64 was considered negative, whereas a titer greater than or equal to 64 was consistent with vaccination or exposure to *B. burgdorferi* (*B. burgdorferi* C6 peptide tests[28] to discriminate vaccination from prior infection were not pursued since dogs were not likely to have been vaccinated); and *R. rickettsii* (immunofluroescent assay), a titer of greater than 64 was considered positive, with a single titer of greater than or equal to 1024 suggesting recent infection, otherwise a positive titer only indicated past exposure or possible crossreactions with another *Rickettsia*. A 4-fold rise in titer 2 or 3 wk after the initial blood sampling was supportive evidence for recent infection. In addition, the 17 dogs for which serum remained after laboratory submission were tested for antibodies against *A. phagocytophilum*; a titer greater than or equal to 80 was considered positive. Screening for antibodies to other *Ehrlichia* or *Bartonella* spp. was not included.

PCR analysis. Approximately 200 µl of EDTA-anticoagulated whole blood and 5-µm-thick formalin-fixed paraffin-embedded tissue sections were used for DNA preparation (DNA MiniKit, Qiagen, Valencia, CA). The only tissues evaluated in this study were lung, spleen, liver, and kidney obtained after experimental endpoint euthanasia; other tissues (including skin, heart, and synovium) were not evaluated owing to research priorities or other lack of availability. PCR was performed with genus-broad or species-specific primers or both. Initial screening for *Anaplasmataceae* genera and species known to infect canines (*E. canis*, *E. chaffeensis*, *E. ewingii*, *A. platys*, *A. phagocytophilum*) was performed by using genus-specific primers, but only specific primers for amplification of available *E. canis*, *E. chaffeensis*, and *A. phagocytophilum* DNA was performed, as previously described.[3,8] PCR for *A. phagocytophilum ankA*, *msp2*, and *rrs* were performed on all samples as previously described.[6-8,32] *B. burgdorferi* PCR was performed on all blood and tissue samples, as previously described.[24] *R. rickettsii* PCR was not performed in this investigation because positive-control DNA was not available at the time of this investigation and because of the acute nature of this infection in dogs. Agarose gel electrophoresis and ethidium bromide staining were used to assess the PCR products. Positive controls included DNA from *E. canis*, *E. chaffeensis*, *A. phagocytophilum*, and *B. burgdorferi* cultures maintained in the laboratory. The negative control sample was water rather than template DNA.

Statistical analysis. Two-tailed Student *t* tests (STATA statistical software, version 8.0, College Station, TX) were selected to assess differences in CBC and biochemistry parameters between seropositive and seronegative dogs for each of the 3 pathogens. In addition, dogs seropositive for all 3 pathogens were compared with those seronegative for all 3 pathogens. Differences were considered significant at a P level of less than 0.05.

Table 1. Serologic, serum biochemical, and CBC results of individual dogs

Dog	*E. canis* titer	*Borrelia* titer	*Rickettsia* titer	total no. of positive reactions	globulin (g/dl)	Total protein (g/dl)	albumin: globulin ratio	platelets ($\times 10^3$/µl)	WBC count ($\times 10^3$/µl)	ALT (U/L)	AST (U/L)
Reference range	≥20	≥64	≥64		1.6–3.6	5.0–7.4	0.8–2.0	170–400	4.0–15.5	12–118	15–66
03-037	<20	0	0	0	2.7	6.1	1.3	46	10.2	89	33
03-036	<20	0	0	0	3	6.2	1.1	234	16	24	27
02-362	<20	0	0	0	3.4	6.3	0.9	185	12.1	24	26
03-010	<20	0	0	0	3.5	6.9	1	117	12.3	43	27
02-299	<20	0	0	0	5.1	8.3	0.6	243	10.5	18	21
02-301	<20	0	0	0	7.5	11.7	0.6	214	16.6	36	45
03-013	<20	512	0	1	2.5	6	1.4	380	14.2	40	26
03-014	<20	512	0	1	3.4	7.2	1.1	387	10.1	39	26
03-051	<20	128	0	1	3.5	6.8	0.9	116	11.3	20	32
03-015	640	128	0	2	3.7	6.7	0.8	400	16.4	38	23
02-319	80	128	0	2	3.9	7.3	0.9	271	13.3	29	31
03-031	40	128	0	2	3.9	7.2	0.8	463	16.4	14	28
03-012	20	256	64	3	4.3	7.5	0.7	234	14.2	39	29
03-011	<20	0	128	1	3.5	6.8	0.9	216	9.8	38	26
03-050	<20	0	128	1	3.9	7.5	0.9	283	15.4	37	23
02-363	160	0	128	2	3.5	7.1	1	196	9.3	26	28
02-302	20	0	256	2	2.9	5.7	1	120	18.3	96	38
02-320	10240	0	256	2	4.6	7.4	0.6	191	13.2	33	58
03-022	320	256	256	3	4.2	7.3	0.7	64	18	15	20
02-283	160	128	256	3	4.6	7.5	0.6	266	22.3	102	30
03-009	80	0	16384	2	4	7.8	1	384	10.3	40	16
no. of dogs positive	10	9	9	15							
no. tested	21	21	21	21							
% positive	48%	43%	43%	71%							

ALT, alanine aminotransferase; AST, asparagine aminotransferase; WBC, white blood cells

Results

CBC and biochemistry analysis. Results of CBC and biochemical analyses for individual dogs are shown in Table 1. Comparisons of serum biochemical and CBC results between seropositive and seronegative dogs are shown in Table 2. Several dogs had elevated WBC counts whereas others had low platelet counts, although this result was not associated with seropositivity or clinical disease. Platelet counts fell below normal range in several dogs, most of which were seropositive for 1 or more pathogens (Table 1).

Compared with published normal values, elevated globulin levels accompanied by elevated total protein and decreased albumin:globulin ratios were present in 52% of dogs, but these features were not consistently related to seropositivity or clinical disease. However, albumin:globulin ratios were decreased consistently in animals simultaneously seropositive for all 3 pathogens compared with those simultaneously seronegative for all 3 pathogens ($P < 0.045$). Albumin:globulin ratios were not different among animals seropositive for 2 or fewer bacteria. Hepatic transaminase and renal function parameters also did not differ between seropositive and seronegative dogs.

Throughout the study, only 2 dogs exhibited clinical signs (including fever, lethargy, and abnormal coagulation studies) suggestive of rickettsial or ehrlichial disease: 1 dog had mild epistaxis, and the other had severe hematoma formation at a venipuncture site. Both dogs were seropositive for all 3 pathogens, had resolving but consistently positive titers after 4 wk (data not shown), and had globulin levels above 4.0 g/dl. One dog was markedly thrombocytopenic, with a platelet count of 64,000/µl. Results for these dogs (02-283 and 03-022) are shown in Table 1. Both were treated with antibiotics, although dog 02-283 was treated with doxycycline for suspected tickborne infection, whereas dog 03-022 was treated with amoxicillin and sulfonamides as part of the cardiac study protocol.

Serology. Of 21 dogs tested, 48% had antibodies to *E. canis*, 43% to *B. burgdorferi*, and 43% to *R. rickettsii*, with an overall prevalence of 71% of dogs positive for one or more pathogens (Table 1). In addition, 7 dogs (33%) had antibodies to 2 pathogens, whereas 3 dogs (14%) had antibodies to all 3 agents. Further, 7 of the 17 dogs (41%) tested had antibodies to *A. phagocytophilum*, with titers of 80 or higher (data not shown). Crossreactivity with *E. chaffeensis* (not tested) is possible because this pathogen also infects dogs. *E. chaffeensis* crossreactivity should be the same as that of *E. canis* because these 2 pathogens are antigenically very similar. However, fewer dogs were seropositive for *A. phagocytophilum* than for *E. canis*, thus suggesting a reduced likelihood of crossreactivity between these 2 pathogens in this study.

PCR analysis. All PCR analyses using primer sets for detection of *Anaplasmataceae* were negative in canine blood and tissues, including liver, lung, kidney, and spleen. In addition, PCR results were negative for *B. burgdorferi* in blood and tissues,

Table 2. Effect of serologic status on select hematologic and biochemical parameters among study dogs

	Ehrlichia canis			Borrelia burgdorferi			Rickettsia rickettsii		
	Seropositive (n = 10)	Seronegative (n = 11)	*P*	Seropositive (n = 9)	Seronegative (n = 12)	*P*	Seropositive (n = 9)	Seronegative (n = 12)	*P*
Globulin (g/dl)	3.96 (0.52)	3.82 (1.40)	0.76	3.78 (0.61)	3.97 (1.31)	0.665	3.94 (0.56)	3.84 (1.33)	0.81
Total protein (g/dl)	7.15 (0.58)	7.25 (1.62)	0.85	7.06 (0.48)	7.32 (1.58)	0.597	7.18 (0.62)	7.22 (1.55)	0.93
Albumin: globulin	0.81 (0.16)	0.97 (0.25)	0.09	0.88 (0.24)	0.91 (0.22)	0.764	0.82 (0.17)	0.95 (0.25)	0.20
WBC ($\times 10^3$ cells/μl)	15.17 (3.92)	12.59 (2.53)	0.09	15.13 (3.67)	12.83 (3.05)	0.133	14.53 (4.43)	13.28 (2.56)	0.47
Platelets ($\times 10^3$ cells/μl)	258.9 (126.43)	220.09 (105.43)	0.45	286.78 (134.57)	202.42 (86.05)	0.096	217.11 (92.78)	254.67 (130.32)	0.42
AST (U/L)	30.10 (11.56)	28.36 (6.48)	0.67	27.22 (3.90)	30.67 (11.51)	0.350	29.78 (12.32)	28.75 (6.18)	0.82
ALT (U/L)	43.20 (30.82)	37.09 (19.35)	0.59	37.33 (26.48)	42 (24.80)	0.683	47.33 (30.37)	34.50 (19.68)	0.25

ALT, alanine aminotransferase; AST, asparagine aminotransferase; WBC, white blood cells
Data presented as mean (1 SD).
P values based on Student *t* test with adjustment for unequal variances, if necessary.

although spirochetemia is not readily detected in blood because of the short period and low level of circulation for spirochetes. False-negative results from tissues were possible because many dogs received antibiotics (amoxicillin or sulfonamides or both) perioperatively. False-negative results from blood were unlikely because blood was taken before experimental manipulations.

Discussion

The risk to dogs for exposure to tickborne pathogens varies with breed, recreational activity, and consistency of flea and tick infestation control.[33] Class B random-source dogs predominantly are hound breeds previously used as hunting dogs. These hound breeds are at high risk of acquiring tickborne infections due to occupational exposure to ticks and other ectoparasites.[23,25] The 3 tickborne pathogens examined here represent those commonly acquired by dogs and other important tick- and ectoparasite-borne pathogens such as *A. platys*, *E. ewingii*, *Babesia canis*, and *Bartonella* spp.[9] were not studied. Each of these pathogens is capable of producing disease that could confound experimental research in the absence of acute active infection and direct pathogenicity, through immune stimulation and hemodynamic alterations that can occur with low-level persistence. This potential effect is particularly likely for *E. canis* and *B. burgdorferi*.

Active infection of dogs seropositive for *E. canis* could not be confirmed in our samples. Presumably the seroreactivity reflects either previous infection, followed by clearance of infection and suppression by perioperative antibiotics after surgical instrumentation (only if doxycycline was used), or persistence below the detection sensitivity of our qualitative PCR. In fact, persistence of *E. canis* is well recognized and can be very difficult to detect.[22] *E. canis* reactivation after immune suppression, stress, or cardiac cachexia[20,36] can lead to overt disease.[22]

Likewise, vaccination for *B. burgdorferi* can confound Lyme disease diagnosis because the vaccine induces antibodies detected by standard laboratory tests. Diagnostic tests using the invariant C6 peptide of *B. burgdorferi* VlsE are recommended instead of classic serology detection because this peptide is not a component in recombinant protein vaccines. Because of the low likelihood that these dogs were vaccinated for *B. burgdorferi*[16] and because of the retrospective nature of this investigation, C6 peptide testing was not performed. Despite the frequency of *B. burgdorferi* seropositivity, the lack of PCR detection of *B. burgdorferi* in blood is not surprising because this assay is insufficiently sensitive to detect spirochetemia levels usually achieved by this bacterium, which preferentially disseminates into tissues (including skin, heart, synovium) in dogs.[2,16,27,29,34,35] Because this bacterium can cause myocarditis and cardiac arrhythmia, even serologic evidence of infection raises concern about confounded scientific studies in canine cardiac disease models.[27] However, 95% of dogs exposed to *B. burgdorferi* do not develop clinical signs,[29] although experimental coinfections with other tickborne pathogens, for example *A. phagocytophilum*, could increase the frequency of clinical signs, such as lameness,[34] and perhaps promote persistence.[35]

Whether dogs exposed to *R. rickettsii* can be infected subclinically is under debate, and many experts contend that Rocky Mountain spotted fever is always acute and moderately severe to severe.[5,12,18] Although seroreactivity suggests exposure, it does not always correlate with disease,[4,26] perhaps owing to tick-transmitted infection by antigenically related *Rickettsia* spp. such as *R. parkeri* and *R. amblyommi*, which are of low pathogenicity and induce serologic responses that cannot be distinguished from those toward *R. rickettsii* by commercial assays.[4,5,26] Whether *Rickettsia* of low pathogenicity can establish persistent infection in dogs and contribute to chronic immune stimulation and sequelae has not been investigated.

Most hematologic and biochemical parameters did not differ significantly between seropositive and seronegative dogs, and many values were within normal published ranges. This pattern was not the case with hyperglobulinemia, for which elevated levels were the predominant explanation for low albumin:globulin ratios that suggest chronic immune stimulation.[17,19] This effect was significant ($P < 0.045$; data not shown) in dogs infected with *E. canis*, *B. burgdorferi*, and *R. rickettsii* compared with those seronegative for all 3 pathogens. Many pathogens can cause chronic immune stimulation, but a comprehensive evaluation of all possible etiologies in these animals was beyond the scope of the study. Histopathologic lesions in kidneys and liver also can occur with chronic immune stimulation and illustrate the caution required in interpretation of tissue lesions in scientific studies.[13,14] These tickborne infections can cause tissue injury and disease that may not be readily detectable by routine laboratory evaluations. Beagle dogs experimentally infected with *E. canis* develop transient proteinuria, predominantly albuminuria, and histopathologic renal interstitial changes characterized by lymphoplamsacytic infiltrates.[10,14] Although these beagles show

few glomerular lesions histopathologically, ultrastructural examination demonstrates minimal-change glomerulopathy and fusion of podocyte processes.[10] In addition, exposure to these pathogens can expand the variability in hematologic, biochemical, and histopathologic parameters and are less likely to reveal subtle differences, ultimately requiring increased numbers of dogs to achieve statistical significance.

All 3 pathogens examined here are zoonotic, can cause human disease, and are the most frequent tickborne agents in humans. During 2005, 21,304 cases of Lyme disease, 1843 cases of Rocky Mountain spotted fever, and 1284 cases of ehrlichiosis (granulocytic anaplasmosis due to *A. phagocytophilum* and monocytic ehrlichiosis due to *E. chaffeensis*) were reported in humans.[15] To diminish human exposure, dogs should be treated with parasiticides to assure killing of all potentially infected tick vectors.[11] However, because many seropositive dogs spontaneously clear infection, humans exposed to blood from dogs by needle sticks or through mucous membrane contact, bites, or scratches are at low risk of accidental infection. A solution for reducing the risk of tickborne infection in research dogs is the repeated use of parasiticides, both at the vendor facility and upon arrival at biomedical institutions. Substantial reduction of the risk of human and animal exposure to these pathogens in the research environment is easily achieved through the use of Class A purpose-bred dogs.

In conclusion, this investigation supports caution among biomedical research stakeholders who are concerned about the unknown health history of randomly acquired research dogs and the impact underlying infections or disease may have on the integrity of scientific data generated from canine models. This retrospective analysis raises many questions that are not addressed here but which could be answered in a carefully planned prospective study. Several important goals include the use of quantitative real-time PCR to enhance sensitivity of pathogen detection in blood and tissue; broader serologic testing to assess potential crossreactivity and to more clearly identify specific pathogen reactivity; increased sample numbers to improve statistical power; collection of blood and tissues before antibiotic therapy to maximize pathogen detection; use of histopathology and immunohistochemistry to identify pathogens in tissue sections; and lastly sequential collection of blood and other clinical samples from each dog to monitor kinetic changes in pathogen load and dissemination, especially during persistent infections.

This investigation examines only a small number of possible etiologies focused on tick-borne infections. The results provide research investigators and laboratory animal veterinarians with clues for identifying tickborne infections and other veterinary conditions that could confound and ultimately compromise scientific results and interpretations in the research environment.

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