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Ehrlichia canis in dogs experimentally infected, treated, and then immune suppressed during the acute or subclinical phases

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Abstract

Background: Concerns for recrudescence of *Ehrlichia canis* infection arise when immunosuppressive drugs are used to treat immune-mediated diseases in dogs previously infected with *E. canis*.

Objectives: Determine whether administration of prednisolone and cyclosporine would reactivate *E. canis* infection in dogs previously treated with doxycycline during the acute or subclinical phases.

Animals: Seven beagles previously experimentally infected with *E. canis* and administered doxycycline for 4 weeks were included. Three of the 7 dogs were incidentally concurrently infected with *Anaplasma platys* and *Babesia vogeli* and were administered 2 doses of imidocarb 2 weeks apart before enrollment in the current study.

Methods: Experimental study. Each dog was administered prednisolone and cyclosporine for 6 weeks. Clinical signs, complete blood cell count (CBC), polymerase chain reaction (PCR) assays for *E. canis*, *A. platys*, and *B. vogeli* DNA in blood, *E. canis* indirect fluorescent antibodies (IFA) titers, and flow cytometry for antiplatelet antibodies were monitored.

Results: All dogs completed the immunosuppressive protocol. No evidence for recrudescence of *E. canis*, *A. platys*, or *B. vogeli* were detected based on clinical signs or results of CBC, PCR, IFA, and flow cytometry for antiplatelet antibodies. *E. canis* IFA titers were negative in 5/7 dogs at the end of immunosuppressive protocol and were negative 6 months after the protocol in 5/5 dogs available for testing.

Conclusions and Clinical Importance: Dogs administered with a 4-week course of doxycycline with or without imidocarb failed to show evidence of activation of *E. canis* infection after administration of a commonly used immune suppressive protocol.

KEYWORDS

Anaplasma platys, *Babesia vogeli*, cyclosporine, *Ehrlichia canis*, prednisolone

1 | INTRODUCTION

Ehrlichia canis is an obligatory intracellular pathogen that causes canine monocytotropic ehrlichiosis (CME), a disease of worldwide

Abbreviations: BSA, bovine serum albumin; CME, canine monocytotropic ehrlichiosis; HCT, hematocrit; IFA, immunofluorescent assay; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PE, physical examination; PRP, platelet-rich plasma.

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importance in dogs.[1] The brown dog tick, *Rhipicephalus sanguineus* is the primary vector for *E. canis* transmission.[2]

Doxycycline is considered the first-line therapy for treatment of CME and a 4-week course administered at 10 mg/kg/day is currently recommended.[3] However, there are discrepant results with regard to clearance of *E. canis* infection in dogs after the recommended doxycycline regimens.[4-6] While 3 or 4 weeks of administration of doxycycline cleared *E. canis* DNA from the blood, bone marrow, spleen, liver, and lungs,[4] *E. canis* DNA was detected in *R. sanguineus* fed on dogs with *E. canis* previously treated with a 4-week course of doxycycline, suggesting persistence of infection.[5]

Because of the inconsistent results regarding the clearance of *E. canis* infection after doxycycline treatment, concerns for recrudescence of disease arise when use of immunosuppressive drugs is indicated for treatment of an immune-mediated disease in a previously infected dog. Glucocorticoids and cyclosporine have been classically used to treat inflammatory or immune-mediated diseases in dogs and both drugs suppress T lymphocyte function.[7-10] Because T lymphocytes are important for protective immunity in ehrlichial infection[11] the disease could potentially be reactivated during immunosuppressive treatment. There was no recrudescence of *E. canis* infection in 2 studies using a short course of dexamethasone (0.4 mg/kg/IV for 1 day or 0.3 mg/kg/IM for 5 days although it is possible the duration of the immunosuppressive protocols were too short to reactivate the infection.[4,12]

The purpose of this study was to investigate whether administration of prednisolone and cyclosporine for 6 weeks would reactivate *E. canis* in previously infected dogs that had undergone doxycycline treatment with or without imidocarb treatment. The primary hypothesis was that administration of prednisolone and cyclosporine in a commonly used protocol would induce reactivation of *E. canis* despite pretreatment with doxycycline with or without imidocarb.

2 | MATERIALS AND METHODS

2.1 | Animals

This project was approved by the Colorado State University Institutional Animal Care and Use Committee. Seven healthy beagles (age: 1 to 2-year-old, weight: 12 to 16 kg) that were laboratory reared and housed in a tick and flea free facility were used in the study. All dogs were fully vaccinated. Abnormalities on complete blood cell count (CBC) or serum biochemistry panel were not detected before this study. All the dogs were negative for DNA of select vector borne disease agents (*Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., hemotropic *Mycoplasma* spp., *Rickettsia rickettsii*, and *Neorickettsia risticii*) using a panel provided by commercial laboratory (Canine tick borne disease profile, ANTECH Diagnostics, Fountain Valley, California). In addition, the dogs were negative for antibodies against *Anaplasma* spp., *Borrelia burgdorferi*, and *Ehrlichia* spp. as well as *Dirofilaria immitis* antigen by use of a commercially available kit (IDEXX SNAP 4Dx Plus Test, Westbrook, Maine).

2.2 | *E. canis* PCR assay

The experimentally infected dogs were also monitored for the presence of *E. canis* DNA in blood using a previously published assay that is provided in an American Association of Veterinary Laboratory Diagnosticians certified laboratory (Specialized Infectious Diseases Laboratory, Veterinary Diagnostic Laboratories, Colorado State University, Fort Collins, Colorado).[13] Briefly, a commercial kit (QIAamp DNA blood mini kit, Qiagen, Chatsworth, California) was used to extract DNA from 200 μL of EDTA anticoagulated blood. Amplification of DNA was performed in a 50 μL reaction mixture containing 5 μL of template DNA; 200 μM (each) dATP, dCTP, and dGTP; 400 μM deoxyuridine triphosphate (dUTP); 1 μM of each 16S-23S intergenic spacer primer (5′ CTG GGG ACT ACG GTC GCA AGA C 3′-forward; 5′ CTC CAG TTT ATC ACT GGA AGT T 3′-reverse); 3.5 mM MgCl2; and 2.5 units of DNA polymerase (Amplitaq Gold, PE Applied Biosystems, Foster City, California) in a 1× reaction buffer (50 mM KCl and 10 mM Tris HCl; pH, 8.3). Amplification was performed by use of dUTP to prevent carryover of the amplified PCR product. Amplification of DNA was performed in a thermal cycler (GeneAmp PCR System 9700 thermal cycler, PE Applied Biosystems, Foster City, California); a timed-release PCR protocol was used. The thermocycle profile consisted of incubation at 20°C for 10 minutes; denaturation at 95°C for 10 minutes; and 45 cycles of denaturation at 95°C (1 minute), annealing at 60°C (1 minute), and extension at 72°C (30 seconds) with a 1-second increment/cycle. Products of PCR were separated via electrophoresis on a 3.0% agarose gel; the gel was stained with EZ-Vision (EZ-VISION, VWR, Radnor, Pennsylvania) and examined under UV light. All appropriate controls are performed on all assay runs. The complete standard operating procedures is available on request (Michael Lappin).

2.3 | *E. canis* indirect fluorescent antibodies (IFA)

The IFA procedure for detection of antibodies against *E. canis* that was used to monitor the experimentally infected dogs in this project is that which provided in an American Association of Veterinary Laboratory Diagnosticians certified laboratory (Specialized Infectious Diseases Laboratory). The complete standard operating procedures is available on request (ML). In this assay, canine samples are diluted from 1:20 with doubling dilutions in 0.075% Tween 20 phosphate-buffered saline (PBS) (v/v) and tested to the highest dilution giving a positive titer. Test controls included seropositive and seronegative sera obtained from a previous *E. canis*-infected dog and a noninfected control dog. Diluted sera was added to a commercially available *E. canis* IFA slides (ProtaTek Reference Laboratory, Mesa, Arizona), incubated 30 minutes at 37°C in a prewarmed, humidity chamber. The slides then were rinsed with 0.075% Tween 20 PBS and placed on rocker in PBS wash for 5 minutes, followed by a distilled H_2O wash for additional 5 minutes. A fluorescein-labeled goat anticanine immunoglobulin G (IgG) antibody (Fluorescein-labeled goat anticanine immunoglobulin G antibody, ProtaTek Reference Laboratory)

diluted 1:40 was added to each well, incubated at 37°C for 30 minutes, and then washed. The slides were rinsed with 0.075% Tween 20 PBS and repeated 5 minutes washes as before. A few drops of mounting fluid were added to slides and glass cover slips covered the slides. Immunofluorescence was detected with a fluorescence microscope. The complete standard operating procedures is available on request (Michael Lappin; Specialized Infectious Diseases Laboratory).

2.4 | Direct flow cytometry for detection of antibodies against platelets

The protocol used for this study was modified from other protocols previously described in the literature.[14,15] Briefly, 500 μL of EDTA anticoagulated blood was mixed with an equal volume of sterile PBS. This mixture was centrifuged at 200*g* for 1 minute 30 seconds at 20°C to generate platelet-rich plasma (PRP). PRP was removed from the erythrocyte layer and placed into an Eppendorf tube. Each PRP sample was adjusted to 2 x 10^6 cells/mL using a manual hemocytometer to provide a standard volume of PRP that was then pelleted by centrifugation at 1000*g* for 5 minutes at 20°C. The platelets were resuspended and washed 3 times at the same speed in a solution containing 3 mM EDTA, 1% bovine serum albumin (BSA), and PBS. Each sample was incubated at room temperature protected from light with 50 μL of a 1:200 dilution of FITC-labeled rabbit anti-dog IgG (FITC-conjugated AffiniPure rabbit anti-dog IgG [H + L], Jackson ImmunoResearch Labs, West Grove, Pennsylvania) for 30 minutes. After incubation, the platelets were washed 3 times with PBS-EDTA-BSA solution and resuspended in 200 μL of PBS-EDTA-BSA solution for flow analysis. Gate settings used for this study were previously established with PE-labeled mouse anti-human CD61 (Anti-human CD61 [Integrin beta 3] PE, VI-PL2, eBioscience, San Diego, California) using healthy dog samples. Platelets from a healthy beagle served as a negative control at each time point. Samples were considered positive if the percent IgG was ≥10%. All samples were analyzed using a Cyan ADP instrument (Cyan ADP instrument, Beckman Coulter, Miami, Florida) and the generated data were analyzed using FlowJo software (FlowJo software, Tree Star, Ashland, Oregon).

2.5 | Experimental inoculations

Four beagles (Group A) were inoculated IV with 1 mL of blood containing *E. canis* DNA that was collected 36 hours previously from a client-owned dog that resided in Todos Santos, Baja California Sur Mexico. Three beagles (Group B) were inoculated IV with 1 mL of blood containing *E. canis* DNA collected within 2 hours from a research beagle that had originally been inoculated with 3 mL of blood from a different dog from Todos Santos, Baja California Sur Mexico that was also positive for *E. canis* DNA. The donor beagle that had been inoculated for use with Group B had not developed significant signs of disease or required treatment. In each of the inoculations, blood was placed in ethylenediaminetetraacetic acid anticoagulant and was transported on cold packs or stored at 4°C until administered. Both blood samples used in the primary inoculations were reported to be below the CT threshold for a positive test result at the commercial laboratory using a fluorogenic PCR assay (Canine tick borne disease profile, ANTECH Diagnostics) and contained adequate DNA for sequencing performed after DNA amplification in the conventional PCR assay Specialized Infectious Diseases Laboratory). The *E. canis* DNA sequences from the dogs used in this study were 100% homologous with GenBank KJ459920.1 (forward and reverse) and 98% homologous with KJ459919.1 (forward) and 97% homologous with KJ459920.1 (reverse), for Group A and Group B dogs, respectively. Blood from the infected dogs is potentially available on request (ML).

2.6 | Monitoring after inoculation

Each dog had a physical examination and blood was collected for a CBC, serum biochemistry panel, the commercially available vector borne PCR assay panel (Canine tick borne disease profile, ANTECH Diagnostics), the conventional *E. canis* PCR assay (Specialized Infectious Diseases Laboratory), the commercially available serological kit (IDEXX SNAP 4Dx Plus Test), the *E. canis* IFA, and flow cytometry for antibody detection on platelets.[14,15]

E. canis DNA was amplified from the blood of all 4 dogs in Group A. The blood was taken on 3 different days approximately 1, 2, and 4 weeks after the inoculation and *E. canis* DNA was repeatedly amplified from the blood. By 2 to 4 weeks after the inoculation, the 4 dogs had *E. canis* IFA titers greater than 1:40, were positive in the commercially available kit for *Ehrlichia* spp. antibodies (IDEXX SNAP 4Dx Plus Test), and were positive for anti-platelet antibodies. Antibodies for *Anaplasma* spp. and *B. burgdorferi* were not detected in any of the Group A dogs. All 4 dogs developed clinical signs including fever, lethargy and anorexia as well as moderate to marked thrombocytopenia (median: 41 x10^3/uL, range: 22 to 92 x10^3/uL).

E. canis DNA was amplified from the blood of all 3 dogs in Group B. The blood was taken on 3 different days approximately 1, 2 and 4 weeks after the inoculation and *E. canis* DNA was repeatedly amplified from the blood. By 2 to 3 weeks after the inoculation, the 3 dogs had *E. canis* IFA titers greater than 1:40, were positive in the commercially available kit for *Ehrlichia* spp. antibodies (IDEXX SNAP 4Dx Plus Test), and were positive for anti-platelet antibodies. Unexpectedly, *B. vogeli* and *Anaplasma platys* DNA were amplified from the blood taken approximately 2 and 4 weeks after the inoculation in the 3 dogs (Canine tick borne disease profile, ANTECH Diagnostics). However, 1 of the dogs did not develop antibodies against *Anaplasma* spp. using the commercially available kit (IDEXX SNAP 4Dx Plus Test). The PCR assay panel performed approximately 1, 2, and 4 weeks after the inoculation for DNA of the other vector-borne diseases and *B. burgdorferi* antibodies were not detected by the commercially available kit. None of the 3 dogs in Group B developed obvious clinical signs related to the vector-borne agents but each developed marked thrombocytopenia (median: 31 × 10^3/μL, range: 28-34 × 10^3/μL).

2.7 | Treatment for vector-borne diseases

The dogs in Group A were administered doxycycline (5 mg/kg PO q12h) for 4 weeks starting approximately 6 weeks after the primary inoculation. The dogs in Group B were administered 2 doses of imidocarb at 6.6 mg/kg IM once separated by 2 weeks for possible *B. vogeli* infection in addition to the 4 week doxycycline protocol starting approximately 10 weeks after the primary inoculation. The PCR was positive for *E. canis* DNA in blood 1 week before the treatment of doxycycline with or without imidocarb in all dogs. The PCR was negative for *B. vogeli* and *A. platys* in blood 1 week before the treatment of doxycycline with imidocarb in all dogs in Group B.

2.8 | Immunosuppressive protocols

The immunosuppressive protocols were initiated approximately 2 months after completion of the doxycycline with or without imidocarb treatment. Each dog was administered prednisolone 15 mg (0.94-1.25 mg/kg) and cyclosporine (Atopica, Novartis Animal Health US, Inc, Greensboro, North Carolina) 70 mg (4.4-5.8 mg/kg) PO q12hr on an empty stomach for 6 weeks. The trough concentration of cyclosporine was measured by a commercial laboratory (Clinical Pharmacology Laboratory, College of Veterinary Medicine, Auburn University, Alabama).[16] The dose of cyclosporine was adjusted based on trough concentration (target trough level: >250 ng/mL) measured on weeks 1, 4, and 6. Prednisolone was tapered down over 2 weeks (0.5 mg/kg once a day for 1 week, then 0.5 mg/kg once every other day for 1 week) before the discontinuation of the administration.

2.9 | Monitoring during and after immunosuppression

The protocol is summarized in Figure 1. Week 0 of immunosuppression was established at the beginning of period when prednisolone and cyclosporine were administered for 6 weeks. Blood was drawn for CBC (weeks 0, 1, 2, 4, and 6), PCR assays for DNA of *E. canis*, *A. platys*, and *B. vogeli* (weeks 0, 1, 2, 4, and 6), flow cytometry for antibody detection on platelets (week 0, 2, 4, and 6), *E. canis* IFA (week 0, 1, 2, 4, and 6), antibody for *Ehrlichia spp*, and *Anaplasma spp* (weeks 0, 1, 4, and 6), cyclosporine trough [weeks 1, 4, and 6 (if the trough on week 4 was lower than a target range)], and serum biochemical panels (pretreatment, week 6). Five out of 7 dogs (2 dogs in Group A and 3 dogs in Group B) were available for blood draw approximately 24 weeks after completing the immunosuppressive protocol (week 30). At that time point, blood was drawn from these dogs for performance of a CBC, PCR assay for *E. canis* DNA, and *E. canis* IFA.

2.10 | Evidence of reactivation

The dogs were monitored clinically twice daily by a veterinarian. A CBC was performed on all days blood draws were performed. Evidence for reactivation of *E. canis*, *A. platys*, or *B. vogeli* was defined by 1 or more abnormalities being present (1) detection of organisms in peripheral blood or DNA amplified by PCR assay; (2) presence of fever, lethargy, or anorexia; (3) CBC abnormalities compared to baseline values and established reference intervals: anemia [hematocrit (HCT) < 35%, reference intervals 40%-55%], moderate thrombocytopenia (< $100 \times 10^3/\mu L$, reference intervals 200-500 $\times 10^3/\mu L$), severe neutrophilia ($30 \times 10^3/\mu L$ <, reference intervals 2.6-11 $\times 10^3/\mu L$) or left shift (bands 300/μL <, reference intervals <200/μL); (4) increase in the percentage of platelets positive for antiplatelet antibodies; and (5) increasing *E. canis* IFA titers.

2.11 | Statistical analysis

The HCT, neutrophils, platelets counts, and IFA reciprocal titers between week 0 and other time points were analyzed by the Kruskal-

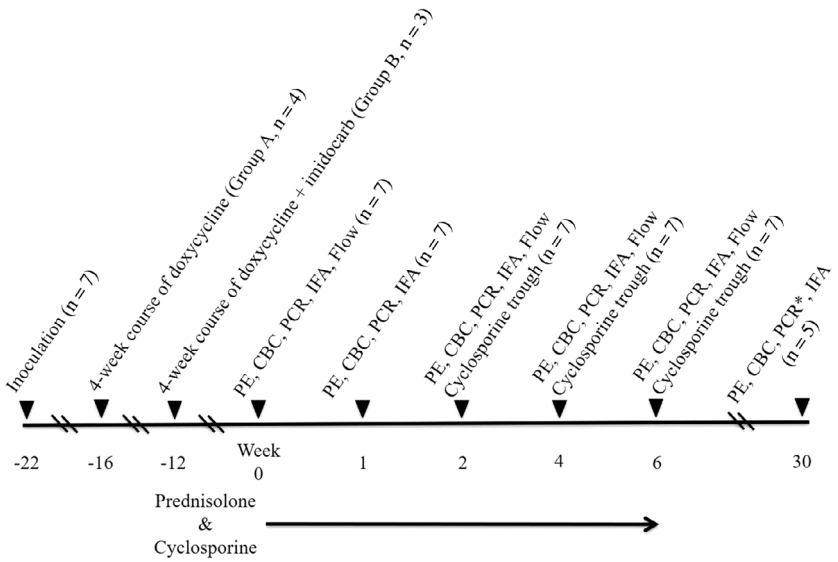

FIGURE 1 Study design. PE: physical examination, PCR: *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., haemotropic *Mycoplasma* spp., *R. rickettsia and N. risticii*, IFA: *E. canis*, Flow: Flow cytometry for antibody detection on platelets. *PCR was performed only for *Ehrlichia* spp. on week 30

Wallis test to assess for differences. Differences of IFA reciprocal titers between week 0 and other time points were evaluated by Dunnett's test. All statistics were calculated by a statistical software (Statmate3, ATMS, Tokyo, Japan). Values of $P < .05$ were considered significant.

3 | RESULTS

3.1 | Dogs before immunosuppression (week 0)

All dogs appeared healthy and had no clinical signs indicative of vector-borne diseases during the week before initiation of the immunosuppressive protocols. The CBC showed thrombocytopenia with occasional clumps in 2 dogs (178 and 197 × 10^3/μL, respectively). All dogs were negative for DNA of *E. canis*, *A. platys*, and *B. vogeli*, and antiplatelet antibody. All dogs had antibody against *E. canis*; the IFA titers ranged from 1:40 to 1:640. Antibodies against *Anaplasma* spp. were detected in 2 Group B dogs.

3.2 | Immunosuppression

All dogs completed the 6-week course of prednisolone and cyclosporine administration without significant adverse events from the medications although 3 dogs developed solitary cutaneous masses consistent with papillomas on the feet. These lesions resolved after stopping the immunosuppressive medications. The median final cyclosporine dose was 6.1 mg/kg twice daily (range 5.3-8.3 mg/kg twice daily). All dogs reached the target cyclosporine trough. The cyclosporine troughs were above the target at 2, 4, and 6 weeks in 2, 3, and 2 dogs, respectively. The median peak trough was 305 ng/mL (range 266-527 ng/mL).

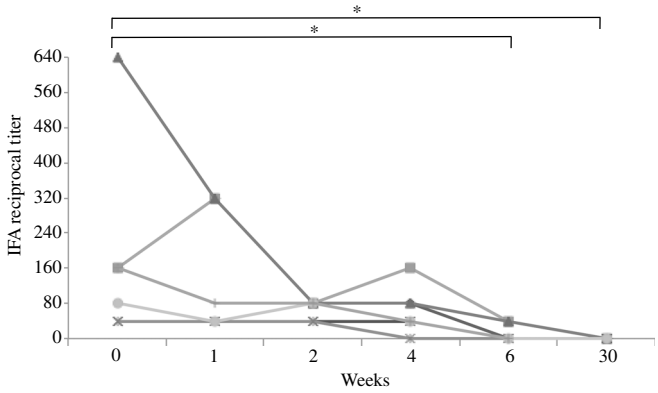

FIGURE 3 *E. canis* IFA reciprocal titers of dogs treated with doxycycline with or without imidocarb before (week 0), during (weeks 1-6), and after (week 30) immunosuppression with prednisolone and cyclosporine. There was a significant difference in the titers between weeks 0 and 6, and 30. *$P < .01$

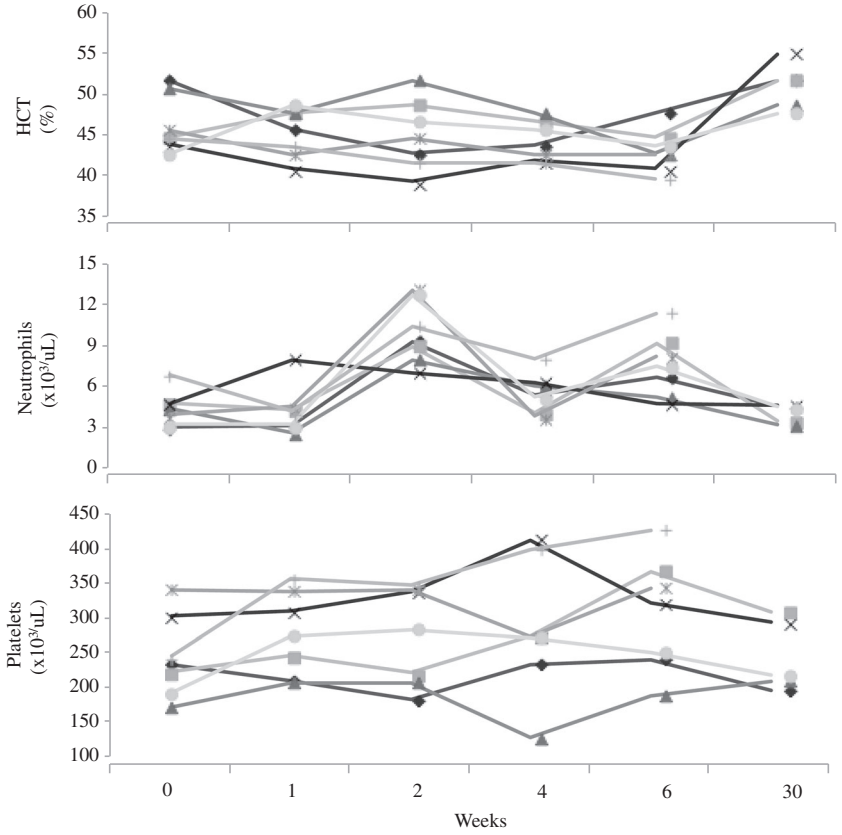

FIGURE 2 HCT (top), neutrophil (middle) and platelet (bottom) counts of dogs treated with doxycycline with or without imidocarb before (week 0), during (weeks 1-6), and after (week 30) immunosuppression with prednisolone and cyclosporine. There were no statistically significant differences in these values between week 0 and other weeks

3.3 | Clinical or laboratory evidence of reactivated vector-borne diseases during or after the immunosuppression

No dogs developed overt clinical signs related to *E. canis*, *A. platys*, and *B. vogeli* during or after the immunosuppressive protocols. HCT, neutrophils, and platelet counts are shown in Figure 2. There were no statistically significant differences in these values between week 0 and other weeks. The PCR for *E. canis*, *A. platys*, and *B. vogeli*, and antiplatelet antibody were negative during the immunosuppression period. All dogs had decreases in the *E. canis* IFA titers during the immunosuppression period (Figure 3). Five out of 7 dogs became *E. canis* IFA negative on week 6 and all 5 dogs that had blood drawn on week 30 had negative *E. canis* IFA titers (Figure 3). Antibody against *Anaplasma* spp. also became negative on week 6 in the 2 dogs that were antibody positive before the immunosuppression.

4 | DISCUSSION

In this study, dogs that had been treated for *E. canis* or *A. platys* and *B. vogeli* infections during the acute or subclinical phases failed to develop clinical or clinicopathological evidence for reactivated infection during a 6-week course of prednisolone and cyclosporine administration. All of the dogs had developed thrombocytopenia, with or without clinical signs (Group A dogs only) that were compatible with acute CME after the primary inoculation. *E. canis* IFA titers were negative in 5 of 7 dogs at the end of immunosuppression and continued to be negative 6 months after the immunosuppression in all 5 dogs that were available for blood draw. Although negative PCR results cannot definitively prove therapeutic elimination of the infection, the negative PCR in conjunction with negative IFA titers and the failure to develop clinical or hematologic abnormalities is highly supportive of therapeutic elimination of the *E. canis* infection in these dogs after the doxycycline with or without imidocarb treatment. It was previously reported that anti-*E. canis* antibodies might persist for months to years even after treatment and resolution of clinical signs.[17,18] In this study, the *E. canis* IFA titers decreased and became negative within 6 weeks after starting the immunosuppression in the majority of dogs. The rapid disappearance of *E. canis* antibody was likely caused by the prednisolone and cyclosporine administration because serum immunoglobulin levels significantly decrease within 2 weeks in dogs given immunosuppressive doses of prednisolone.[19]

Clinical signs related to the *E. canis* infection after inoculation were observed in dogs in Group A but not in Group B. The DNA sequences of the 2 strains of *E. canis* used in this study were not identical and so the organisms might have had different pathogenicity as indicated by the differences in clinical findings in the 2 groups of dogs after primary inoculation. However, the different clinical findings after the primary inoculations in the 2 groups of dogs could also merely relate to different doses of *E. canis* that were likely administered. As *E. canis* is difficult to quantitatively culture and amounts of DNA in a single blood sample do not necessarily correlate to amounts of living *E. canis* in the inoculum, this hypothesis is difficult to prove. However, none of the dogs in either group had evidence of reactivated *E. canis* infection on the immunosuppression protocol.

The dogs in Group B were also incidentally PCR positive for *A. platys* and *B. vogeli* after the inoculation although the donor was PCR negative for these infections in the original screening test. Because negative PCR results cannot rule out the presence of vector-borne diseases it was suspected that the donor was coinfected with *A. platys* and *B. vogeli* because the primary tick vector for *E. canis*, *A. platys*, and *B. vogeli* is *R. sanguineus*.[20] In North America, disease caused by *A. platys* infection alone is considered mild and can resolve with or without treatment, presumably caused by an immune response,[21] whereas there are *A. platys* strains of higher pathogenicity in the Mediterranean countries.[22] Doxycycline is the treatment of choice for *A. platys* infection.[23] *B. vogeli* is a large *Babesia* species that might or might not cause clinical babesiosis depending on the strain and host factors.[24] It is generally accepted that the labeled dose of imidocarb (6.6 mg/kg IM with a repeat dose in 14 days) eradicates *B. vogeli* infection.[25] Doxycycline and imidocarb treatment likely eliminated *A. platys* and *B. vogeli* as well as potentially *E. canis* infections in these dogs, and no increased risk of recrudescence in coinfections was observed.

The timing of doxycycline treatment for CME could affect the treatment efficacy. CME is divided into acute, subclinical, and chronic phases.[5,23,26,27] The acute phase of CME begins approximately 1 to 2 weeks after experimental infection and involves thrombocytopenia, fever, depression, and anorexia.[23,26,27] Clinical signs subside approximately 3 to 4 weeks after the infection, which is usually followed by a subclinical phase that can last from months to years.[23,26,27] Chronic CME is the third phase, which can manifest as mild to severe clinical and hematological abnormalities including weight loss, lethargy, pancytopenia, and monocytosis.[23,26,27] It is reported that chronic CME can be more difficult to treat or clear the infection.[5,23] None of the dogs in this study developed chronic CME during the time studied. A further study is needed to investigate a potential risk for the use of immunosuppressive medications in dogs treated during chronic CME.

Prednisolone and cyclosporine were elected as immunosuppressive agents because both drugs are effective in the control of a variety of immune-mediated diseases in dogs.[7] The dose of prednisolone used in our study was determined based on previous reports and is considered an immunosuppressive dose in dogs.[9] The starting dose of cyclosporine was also determined based on previous studies[28,29] but the cyclosporine absorption in dogs can be variable in each individual, therefore the dose of cyclosporine was adjusted based on the trough levels in our study. The trough level was determined based on therapeutic levels in humans (150-250 ng/mL)[30] because the targeted blood levels of cyclosporine necessary for the effective treatment of immune-mediated diseases are not as clearly established in veterinary medicine. All dogs reached the target trough during the immunosuppression but the timing to reach it varied (the target trough was achieved in 2, 4, and 6 weeks in 2, 3, and 2 dogs, respectively). The cyclosporine might not have taken effect in the 2 dogs that reached the target trough at 6 weeks. Trough whole-blood cyclosporine levels between 400 ng/mL and 600 ng/mL are anecdotally used as the therapeutic target in dogs for efficacy and

safety, although there are no data available that they are correlated with clinical response or immunosuppression.[29] Pharmacodynamic assays such as T-cell cytokine expression measurement could be a better marker to estimate the dose of cyclosporine needed to maintain immunosuppression.[31] Though it was questionable that the dose of cyclosporine used in our study appropriately suppressed T-cell function, the immune system in our dogs was likely impaired because the immunosuppressive dose of prednisolone was used in conjunction with cyclosporine and 3 dogs developed lesions consistent with cutaneous papillomas at the end of study.

In all dogs, serum antiplatelet antibodies were detected after the inoculation, which was consistent with a previous report[32] and they were negative before the immunosuppression after the doxycycline treatment with or without imidocarb treatment. The antiplatelet antibodies were monitored during the study period to evaluate if it could be used as a marker for the reactivation of *E. canis*. The clinical usefulness of monitoring antiplatelet antibodies was unknown in this study because no dogs had reactivated *E. canis* infection.

There were several limitations in this study. First of all, healthy beagles were used, which might not reflect the situation in a clinical setting. Also, dogs were experimentally infected with *E. canis* via needle injection, which is different from a natural infection where tick transmission is the most likely infection scenario. Some reports showed persistent *E. canis* infection after doxycycline treatment in dogs that were experimentally infected via tick transmission or naturally infected that was also likely via tick transmission.[5,6] In addition, as discussed previously, it is likely 2 different strains and doses of *E. canis* were used.

Another limitation to the study is that xenodiagnosis with ticks or PCR performed on splenic and bone marrow aspirates samples, which are considered sensitive methods for detection of *E. canis* infection,[5,33-35] were not utilized in this study. Though the negative PCR and IFA results with no clinical or hematologic abnormalities were highly supportive of elimination of the *E. canis* infection, further evidence of clearance of *E. canis* after treatment would have been provided by use of those additional 2 techniques.

Almost half of the dogs received imidocarb in conjunction with doxycycline that might have synergistic effects against *E. canis* infection and might have affected the results of this study. Lastly, different strains of *E. canis* from our study might show a different response to doxycycline treatment because variations in pathogenicity of different *E. canis* strains exist.[23] Our results might be more applicable to North American strains of *E. canis* because *E. canis* from other areas of the world might have higher pathogenicity.

In conclusion, our study revealed a 4-week course of doxycycline with or without supplemental imidocarb cleared *E. canis* infection induced by IV inoculation for at least 6 months after immunosuppression. Whether these results will be the same in naturally infected dogs will require further study.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

None of the drugs used in this study are labeled for use in dogs except imidocarb for *Babesia canis* infection.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Colorado State University IACUC.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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