

STUDY PROTOCOL FOR VETERINARIANS

Efficacy of Doxycycline Therapy for *Anaplasma phagocytophilum* Infection in Dogs

Thank you for your interests in participating in our study. Your involvement is essential for its success! This protocol summarizes the study, however, **YOU SHOULD CONTACT US PRIOR TO SAMPLE SUBMISSION FOR COORDINATION OF RECEIPT OF THE START-UP PACKAGE**, which includes epidemiological questionnaires, consent forms, sample submission form (for free CBCs at IDEXX labs), tick-preventive products for enrolled dogs and a Fedex account number for free shipping of samples to IDEXX lab and to NCSU lab. **We will not be able to enroll your patient if you have not contacted us before submitting samples.**

Study objectives:

- Evaluate the efficacy of doxycycline for treatment of *A. phagocytophilum* in naturally infected dogs;
- Identify potential chronic infections with *A. phagocytophilum* in naturally exposed dogs.

Design:

Dogs with suspected canine anaplasmosis will be evaluated before antibiotic therapy (day 0), and at 30 and 60 days after the beginning of doxycycline therapy. Dogs should be treated with doxycycline for 28 days. It is vital that samples are collected as close as possible to these dates. **Even if a dog is 100% better following treatment, it should be re-evaluated 30 and 60 days after the beginning of the antibiotic therapy,** since dogs may be a reservoir of this organism.

Steps	Initial Evaluation (day 0)	Doxycycline treatment for 28 days	1 st Follow-up (day 30)	2 nd Follow-up (day 60)
Client questionnaire	Initial questionnaire		Follow-up	Follow-up
Veterinarian questionnaire	Initial questionnaire		Follow-up	Follow-up
Blood-EDTA sample (purple top)	Yes		Yes	Yes
Serum sample (red, gold or tiger top)	Yes		Yes	Yes
Lymph node aspirate	Yes		No	Yes
Complete blood count	Yes		Yes	Yes
Biochemistry panel results (if available)	Yes		Yes	Yes
Urinalysis results (if available)	Yes		Yes	Yes
Apply Advantix® topically and provide product for application in 2 weeks.	Yes		Yes	No End of study

Treatment:

For this study, treatment is based on **DOXYCYCLINE** 5 mg/kg PO each 12 hours for 28 consecutive days.

Important reminder about treatment:

If you will use any other antibiotic for the initial treatment of a dog with suspected or confirmed Canine Anaplasmosis, **please do not enroll this patient in this study.** On the other hand, if you need to change the treatment after the initial 28 days of doxycycline for another antibiotic or combination of antibiotics, don't exclude the patient from the study. Just describe the treatment prescribed in the specific field of the follow-up questionnaire.

Characteristics needed to enroll a dog in the study:

Patients should be enrolled in this study **only if they have at least 3** of the following (table next page):

Inclusion criteria:

Presence of ticks or history of tick infestation within 4 weeks	Acute onset of lethargy or depression	Fever (temperature > 102.9°F)
Joint pain	Neck pain	Bleeding
Anorexia	Vomiting	Neurological signs
Inflammatory ocular disease	Lameness	Anemia (PCV <35%)
Leukopenia (WBC <6,000 cells/μL)	Thrombocytopenia (platelets <200,000 cells/μL)	Hyperproteinemia (TP >7.8 g/dl)

Characteristics to exclude a dog from the study:

- Dogs treated with antibiotics or anti-rickettsial drugs during the 4 week period before this evaluation;
- Dogs which will not be treated with doxycycline, or will be treated with an antibiotic other than doxycycline, or will be treated with doxycycline for a period of less than 28 days;
- Dogs whose owners cannot return for follow-up examinations at 30 and 60 days after the initial evaluation;
- Dogs whose owners do not agree or are not capable of applying the provided tick/flea preventive every 2 weeks;
- Dogs whose owners do not agree with or do not sign the consent form.

Important reminder about inclusion criteria:

Snap 4Dx positive result for *A. phagocytophilum* **IS NOT** an inclusion criterion. Dogs with negative results for Snap test may still be enrolled if they fulfill at least 3 of the inclusion criteria listed above.

Questionnaires:

You will be provided with 2 sets of questionnaires: one for the initial evaluation and one to be used in each follow-up (day 30 and day 60). Each questionnaire is sub-divided into 2 parts: client answers and veterinarian answers. Please, fill out each section at each visit and submit them with the samples and a signed consent form.

Follow-up costs:

Unfortunately we cannot cover veterinary service costs for initial consultations and follow-ups. We count on your collaboration in re-evaluating dogs and collecting samples after treatment. Some veterinarians participating in this study will waive clients' costs of follow-ups if they are intended strictly for sample and data collection for this study. However, this decision is solely based upon your judgment and we appreciate your contribution to obtaining the results that will be generated through this collaborative research study.

Complete blood count:

CBCs are covered by this study. An aliquot of whole blood in EDTA (1 ml at least) from the first evaluation and each of the follow-ups should be sent to: **IDEXX Preclinical Research Services, 3 Centennial Drive, Suite 1, North Grafton, MA, ZIP 01536**, Phone (207) 439-9291. Attn: Anita Pacitti.

There is a specific form to be filled out and submitted with the sample (form "Clinical Samples Inventory Forms A and B"). Any field highlighted in yellow in that form should be filled out. Results will be available up to 72 hours only by email, so be sure you complete the field providing this information. Ship samples overnight on ice packs. We will cover shipping costs. The Fedex account number will be provided in the start-up package. **Be careful that blood for PCR, serum and lymph node aspirates should be shipped to another address**, described on next page.

Biochemistry panel, urinalysis, Coomb's test:

Other blood work tests cannot be covered by this study. We do not require veterinarians to request these tests at the expense of the clients. Our goal is to use any available information from each case if the tests were requested for defining clinical decisions made by participating veterinarians. Thus, we would like to ask you to send with the samples any available laboratory test results obtained at each evaluation time point.

Tick/flea preventive:

We can provide K9 Advantix[®] free of charge (provided by Bayer[®] Animal Health) for clients participating in this study. The goal is to prevent dogs from re-infection during the study. **This is a crucial step!** Failure to control ticks and fleas infestation can ruin this study, because dogs re-infected with *A. phagocytophilum* would be indistinguishable from chronically infected dogs. For this reason, we ask clients to apply the product **every two weeks** during the study. One simple, but important, step is the application of tick preventive during the first evaluation. We request that the veterinarian or veterinary assistant teach the clients to apply the product as indicated by manufacturer. Clients are not obligated to use Advantix[®] if they have special preference for other product; however, the study can not provide or support other tick and flea preventive.

Sample volume for the study, samples storage, packaging and shipping instructions:

- Collect at least 3 ml of whole blood in EDTA and 3 ml of separated serum from each dog in each evaluation. Preferentially, use plastic tubes to avoid broken glass tubes during transport;
- Samples can be stored at refrigerator temperature for 2 weeks before shipping;
- Lymph node aspirates should be performed only at the beginning (day 0) and at the end of the study (day 60);
- Please indicate which lymph node was sampled on each vial;
- Pack samples in leak-proof plastic bags, separated from questionnaires and laboratory test results. Protect tubes with plastic bubble wrap or other appropriate shipping material;
- Ship samples overnight on ice packs. The Fedex account number will be provided in the start-up package;
- The shipping address is:

Pedro Paulo Diniz, 4700 Hillsborough Street, Research Bld, room 462A, Raleigh, NC, 27606.

Protocol for obtaining a lymph node aspirate for the purposes of PCR:

- Before obtaining the aspirate, inject 1 ml of sterile saline into a sterile, unopened red-topped tube. Do NOT use a serum separated tube. Please label the tube with the animal's name, date of sample collection, lymph node aspirate, and which node was aspirated.
- Aspirate lymph node using an 18 to 20 gauge needle and a 12cc syringe for adequate suction.
- Using the same needle and syringe, uptake sterile saline from the sterile red-topped tube into the syringe containing the aspirate. Rinse 3 to 5 times to wash the lymphoid cells from the needle into the collection tube. Do not remove the needle from the tube until the washing is complete.
- After washing, leave the aspirate and saline in the red-topped tube and ship it for further analysis.
- If needed, repeat the aspiration process using a new tube for each additional lymph node.

Turnover of PCR results

Only PCR results will be provided to participating veterinarians during this study, since other tests will be performed in batches. PCR results will allow veterinarians to best direct patient therapy. PCR result from the initial sample (day 0) will be available as soon as possible, but no longer than 4 weeks after submission (the current turnover is less than 1 week). Result from the second sample (day 30) will be provided only when the third sample (day 60) is received in our laboratory. Results will be provided by email, preferentially. Dogs with a negative PCR result will be expected to remain in the study and submit the remaining samples, although therapy may be altered after discussion with the PI.

Authorship:

Veterinarians will be invited to participate as co-author of this study if they enroll at least 30 dogs with full evaluation (days 0, 30 and 60). All other participating veterinarians will be acknowledged in the manuscript.

More information or questions, please contact Dr. Pedro Diniz, (919) 513-8007, Pedro_Diniz@ncsu.edu, or Julie Bradley, (919) 513-8279, Julie_Bradley@ncsu.edu or Dr. Edward Breitschwerdt, (919) 513-8277, Ed_Breitschwerdt@ncsu.edu.