

Dear Participating Veterinarian and/or Participating Owner:

On behalf of Dr. Ed Breitschwerdt and the Vector Borne Disease Diagnostic Laboratory (VBDDL) at North Carolina State University College of Veterinary Medicine (NCSU-CVM), thank you for participating in the study of the “Molecular Epidemiology of *Ehrlichia* and *Bartonella* Species Infection in Golden Retrievers with Lymphoma”.

With your assistance, we hope to clarify the potential role of vector-borne bacteria as co-factors in the development of lymphoma in dogs. Specifically, we hope to provide serological and molecular evidence of infection with *Bartonella*, *Ehrlichia*, and/or *Anaplasma* species in dogs with lymphoma, as well as identify any epidemiological associations or concerns that may not have been previously recognized. We have chosen to limit our study population to Golden Retrievers, as the breed has been shown to be at an increased risk for the development of lymphoma.

We intend to determine whether dogs with lymphoma are more frequently exposed to *Bartonella*, *Ehrlichia*, and/or *Anaplasma* species based on testing of serum for antibodies against *Bartonella* species antigens, against *Ehrlichia canis* antigen, and against *Anaplasma phagocytophilia* antigen. Serological (indirect immunofluorescent antibody, IFA) analysis will be coupled with the testing of whole blood, lymph node aspirates, and buccal/cheek swabs by polymerase chain reaction (PCR) amplification for *Bartonella*, *Ehrlichia*, and *Anaplasma* DNA to determine active infection. This study comprises a prospective, case-controlled, multi-center trial to evaluate 40 dogs with lymphoma for evidence of *Bartonella*, *Ehrlichia*, and/or *Anaplasma* infection; these results will be compared with a control group of 80 dogs that lack clinical evidence of lymphadenopathy.

Enrollment criteria

Cases: Golden Retrievers with a new diagnosis of lymphoma, by cytological or histopathological methods, will be evaluated as case dogs. Our minimum entry criterion for a case is diagnosis of lymphoma by a board-certified clinical pathologist. These cases must not have received any antibiotics within 14 days prior to sample collection (or 30 days for azithromycin) and must not have been previously treated with chemotherapeutics.

Controls: For each case, two to three control dogs will be utilized. Controls should reside within 100 miles of the case dog and ideally be age (\pm 18 months) and sex matched with the case. These control dogs should be healthy and must lack clinical evidence of lymphadenopathy, making the possibility of undetected lymphoma unlikely. Additionally, these control dogs must not have received any antibiotics within 14 days prior to sample collection (or 30 days for azithromycin). These healthy dogs may be identified by the owner of the case dog or selected by the case’s attending veterinarian. If necessary, researchers can work in conjunction with the Golden Retriever Club of America (GRCA) to find appropriate control animals. **If the owner of the case dog or the case’s attending veterinarian cannot identify and recruit healthy control dogs matching the entry criteria, please contact Ashlee Duncan at (919) 513-8279 or (919) 513-8280 at your earliest convenience so that she can contact the GRCA and begin to identify appropriate control dogs.**

Samples: Whole blood (3 to 5 mL in a purple-topped tube), serum (1 to 3 mL in a red-topped tube), lymph node aspirates (in sterile saline), and buccal swabs (1 to 2 swabs/dog) will be submitted to the NCSU-VBDDL. Additionally, copies of the cytology or biopsy report that confirmed a diagnosis of lymphoma and any laboratory test results associated with the case dog’s presentation for lymphoma will be sent to the NCSU-VBDDL. Serum samples will be tested for the presence of antibodies against *Bartonella*, *Ehrlichia*, and *Anaplasma* species, while whole blood, lymph node aspirates, and

buccal swab samples will be examined for the presence of *Bartonella*, *Ehrlichia*, and *Anaplasma* DNA utilizing a sensitive and specific PCR test. Protocols for obtaining lymph node aspirates and buccal swabs are included with this letter.

Paperwork: Using the enclosed consent forms, please obtain client consent for participation in this study. The owner of the dog with lymphoma should sign one consent form, while the owner of the healthy dog(s) should sign the other consent form. We also ask that you or a member of your staff complete the attached questionnaire, as completely as possible, for each sample set you submit. The information provided in the questionnaire will be critical in detecting important clinical or epidemiological trends. Consent forms and questionnaires should be returned with the samples.

We will process samples at no charge to you or your client (a \$360.00 value based on current serology/PCR testing costs in the VBDDL); however, we have no means by which to cover in-clinic expenses for the collection and shipment of samples. **Please refrigerate blood, serum, lymph node aspirates, and buccal swabs for up to 1 week prior to shipment.** Samples should be shipped Monday through Thursday only. Please send the above samples overnight express, packaged appropriately with ice packs and packing material to the following address:

Attn: Ashlee Duncan
Vector Borne Disease Diagnostic Laboratory
NCSU-CVM, Research Building, Room 462A
4700 Hillsborough Street
Raleigh, NC 27606

Test results will be provided to the attending veterinarian; **we ask your patience to allow us the time necessary to collect all data pertaining to each case.** Although we can provide advice and consultation, treatment decisions will be left to the discretion of the attending veterinarian. After the study is complete, the veterinarian will be provided with a summary of the results. We realize that successful completion of the research objectives relies on your judgment and good will. We greatly appreciate the time and effort you will be contributing to identify patients that fit the study criteria, as well as participating in sample collection and submission. Several consent forms and questionnaires for cases and controls have been included for your convenience. Please make additional copies as necessary. I can be reached at (919) 513-8280 or by email at awduncan@ncsu.edu. Furthermore, if you have any questions about this study, please do not hesitate to contact Dr. Breitschwerdt (ed_breitschwerdt@ncsu.edu) or myself.

Thank you for your participation,

Ashlee W. Duncan, MS
Edward B. Breitschwerdt, DVM, DACVIM

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

Note: We would prefer that two lymph nodes be aspirated. Additionally, the procedure for obtaining a lymph node aspirate for PCR is similar to that routinely used for flow cytometry at the NCSU-Veterinary Teaching Hospital, except that sterile saline is used rather than RPMI.

1. Before obtaining the aspirate, inject 1 mL of sterile saline into a sterile red-topped tube. **Please label the tube with the animal's name, date of sample collection, lymph node aspirate, and which node was aspirated.**
2. Aspirate lymph node using a 20 to 22 gauge needle.
3. Using the same needle and syringe, uptake sterile saline from the sterile red-topped tube into the syringe with the aspirate. Rinse 3 to 5 times to wash the cells from the needle into the collection tube. Do not remove the needle from the tube until the washing is complete.
4. After washing, leave aspirate and saline in the red-topped tube for further analysis.
5. Repeat process as needed using a new tube for each additional lymph node.

Protocol for obtaining buccal swab samples for the purposes of PCR:

Note: The procedure for obtaining a buccal swab is similar to that routinely used for culture purposes. Further, this protocol is appropriate for swab collection using Epicentre's Catch-All Collection Swab or a cotton swab.

1. Label the appropriate number of swabs. Use 1 to 2 swabs per animal.
2. Open the package and remove the swab from the packaging. Grasp the handle and pull the swab out; **avoid touching the soft end of the swab.**
3. Hold the dog's head firmly against your body with one hand. With the other hand, place the swab into the dog's mouth against the inside surface of the cheek. Collect tissue by rolling the swab firmly on the inside of the cheek, approximately 20 times on each side, making certain to move the swab over the entire cheek.
4. Place the swab back into the plastic cylinder, leaving the top off to allow the swab to air dry for 10 to 15 minutes at room temperature. **OR** Place the swab into a sterile red-topped serum tube and break off the handle so that the swab will fit completely in the tube. Allow the swab to air dry for 10 to 15 minutes at room temperature. **Avoid touching the soft end of the swab.**
5. After air-drying, place the top on the cylinder/tube. Seal cylinder with a piece of scotch tape or the serum tube with the tube stopper. **Please be certain to label the cylinder/tube with the animal's name and date of sample collection.**
6. Store the air-dried swab in the original plastic container or serum tube in the refrigerator for **up to 1 week** prior to shipment.
7. Repeat the process for subsequent swabs as necessary.
8. To prevent cross contamination, do not allow other dogs to interfere while you are collecting the intended dog. If you plan to collect more than one dog, wash your hands between dogs to prevent any cross-contamination.