Protocol 41056 Amendment 4.1

Approval date	08/18/2023
Expiration date	03/17/2026

1. Basic Information

1. Elements ID

For existing protocols, enter the ID assigned to this protocol in Topaz Elements.

2. eACUC Number (Automatically Assigned)

41056

3. Principal Investigator

Ganta, Roman Reddy

Job title MCKEE ENDOWED PROFESSOR
Department Veterinary Pathobiology
Division Veterinary Medicine
Business unit University of MO-Columbia

4. Protocol Title

Tick-borne rickettsial diseases; pathogenesis and vaccine development

5. Triennial Re-write

Is this protocol a triennial re-write of a protocol that was previously approved at the University of Missouri?

○ Yes ● No

2. Species Section

1. Please note, the total number of animals requested is the amount of animals you will need for a 3 year period. This number should include all experimental animals plus animals used for colony maintenance (breeders and offspring produced that are not used for experiments). These numbers should match the amounts in the Justify Animal Numbers section. If this is a triennial re-write these amounts should also include any animals on the previous protocol that will be transferred to the new protocol.

Species	Strain/ Stock/ Breed	Age/ Weight	Pain/Distress Category	Authorized	Ordered	Received	Adjustment	Available
Cattle	Holstein	6-12 months	Undefined (non-covered species only)	1				1
			USDA Category E	20				20
Total Cattles:				21	0	0	0	21
Dog	Beagle	6-10 months	USDA Category D	346	0	34		312
			USDA Category E	52	0	6		46
Total Dogs:				398	0	40	0	358

2. Phenotypic consequences

Describe any phenotypic consequences of the genetic changes to the animals and the outcome of these consequences (e.g. whether or not any change in animal welfare or husbandry is anticipated).

No Phenotypic consequences...

3. Wild Animals

Are WILD ANIMALS to be used or studied?

○ Yes ● No

4. Client-Owned Animals

Are CLIENT-OWNED animals to be used or studied?

○ Yes ● No

3. USDA Category E

1. Justification for Withholding Drugs

Provide scientific justification for withholding pain/distress-relieving drugs:

Non-vaccinated infection control animals in the RMSF study (project 3) will serve as controls to aiding to differentiate how effective the vaccine will be. If we start giving treatments to the control animals, we will not be able to make true comparisons of vaccine-associated protection, which is the primary goal of the study.

Primary objective of project 4 is is to define Heartwater disease parthenogenesis in the US cattle. We will need to record the infection severity as measured by clinical signs following infection with Ehrlichia ruminantium pathogen. Therefore, offering drugs to reduce the clinical signs will prevent us from assessing the disease severity.

2. Monitoring Pain and Stress

Explain how the level of pain or physical stress will be monitored (include the frequency of monitoring).

We will monitor all animals more closely; twice a day from the time we will fist observe clinical signs. In the event animals begin to show a severe disease symptoms, we will promptly contact the assigned veterinarian for guidance. Accordingly, we may initiate supporting care such offering subcutaneous fluid therapy and/or other care as recommended, but not providing antibiotic treatments.

3. Point of Euthanasia

Define the point at which the animals will be euthanized.

The decision to euthanize animals will be as per the animal health status monitored and subject to recommendation of the veterinarian assigned to the project. Importantly, we will actively seek guidance regarding health status changes of animals and a decision will be made with a high priority given in providing humane treatment of animals.

4. Proposal Overview

1. Purpose

Purpose of the study:

To support the federally funded research grant proposals current in progress:

We currently have three active NIH funded R01 applications involving the use of animals; these studies involve the use of the canine host. We also have an active USDA cooperative agreement grant. This study will involve the use of cattle.

1) NIH R01 grant # AI070908 (title: Vector and host contributions to the regulation of E. chaffeensis gene expression), we will need to perform in vivo screening of Ehrlichia chaffeensis mutants to identify genes essential for pathogens survival in vertebrate and tick hosts.

2) NIH R01 grant # AI152418 (title: Vaccines against Ehrlichia and Anaplasma species infections), canine host will be used to define the value of a modified live vaccine studies protecting against tick-borne rickettsial infections by Ehrlichia chaffeensis, Ehrlichia canis and Anaplasma phagocytophilum. Three primary goals (experiments) of this project are to; 1) evaluate the value of modified live attenuated vaccine (MLAV) to define the duration of immunity against wild type infection challenge through blood stream and tick transmission; 2) determine if immunity to MLAV protects against genetically distinct E. chaffeensis strains; and 3) to evaluate similar MLAVs from related Ehrlichia and Anaplasma species for their usefulness as a live attenuated vaccine protecting against infections. Goals of the first experiment are already accomplished during the year 1 and 2 funding, while experiments 2 and 3 are yet to be accomplished.

3) NIH R01 grant # AI152417 (title: Rocky Mountain Spotted fever vaccine development), we proposed to investigate the utility of whole cell inactivated vaccine to prevent Rocky Mountain spotted fever in dogs. This project major goals (experiments) involve the use of canine host; 1) evaluate inactivation methods for preparing WCA-S (Sheila Smith strain) and adjuvants in defining the vaccine protection; 2) evaluate the duration of immunity; 3) evaluate protection against tick-transmitted challenges; and 4) evaluate WCA protection against R. rickettsii heterologous strain infection challenges.

4) Ehrlichia ruminantium is an important foreign animal disease pathogen of ruminants as the infections with it in non-endemic regions can inflict major morbidity and mortalities. This sub-Saharan African pathogen is also well established in parts of the Caribbean islands. The goals of this proposal are to; 1) investigate heartwater disease pathogenesis in cattle resulting from an important tick-borne foreign animal disease pathogen in ruminants, Ehrlichia ruminantium; and 2) test if E. ruminantium can be transmitted by

Amblyomma maculatum; the tick previously identified as a competent vector and having wide distribution in southeastern parts of the USA.

2. Value

Please provide the information necessary to allow the ACUC to evaluate the objectives of the study against potential animal welfare concerns.

The studies in all four projects are independent and are critical for advancing our understanding of important tick-borne diseases impacting dogs, people and ruminants. The first project goals are to perform mutational analysis and in vivo screening to identify genes essential for the Ehrilchia chaffeensis pathogenesis in vertebrate and tick hosts. The second proposal aims to evaluate modified live vaccines against tick borne diseases in dogs and people resulting from E. chaffeensis, E. canis, and Anaplasma phagocytophilum. The 3rd project evaluates an inactivated whole cell antigen-based vaccine to confer protection against Rocky Mountain spotted fever (RMSF) which is a major fatal disease in dogs and people. The 4th project investigates pathogenesis of an important foreign animal tick-borne disease of ruminants. There are no non-animal alternatives for these tick-borne diseases. The objectives of the studies are the first to define pathogenesis and vaccine development in physiologically relevant animal models. All studies will be performed in accordance with the animal welfare regulations and the studies aim to develop the most effective methods to protect animals from several important tick-borne diseases which are more common in companion animals, agricultural animals and in people.

3. Lay Term Description of Experimental Design

To put something in layman's terms is to describe a complex or technical issue using words and terms that the average individual (someone without professional training in the subject area) can understand. This section should be written so that someone with a **10th grade science education can easily understand the project.**

The studies in all four projects are independent and are critical for advancing our understanding of important tick-borne diseases impacting dogs, people and ruminants. The first project goals are to perform mutational analysis and in vivo screening to identify genes essential for the Ehrilchia chaffeensis pathogenesis in vertebrate and tick hosts. We will generate large pools of E. chaffeensis transposon mutants in support of this objective. The second proposal aims to evaluate modified live vaccines against tick borne diseases in dogs and people resulting from E. chaffeensis, Ehrlichia canis, and Anaplasma phagocytophilum. We recently developed a modified live attenuated vaccine which confers protection against infection challenge by direct blood-borne infection and against tick-transmission challenge. Specifically in the current project, we aim define the protection against heterologous strains of E. chaffeensis, and similarly test homologous modified live vaccines to protect dogs against E. canis and A. phagocytophilum infections. The 3rd project evaluates an inactivated whole cell antigen-based vaccine (WCAV) to confer protection against Rocky Mountain spotted fever (RMSF) which is a major fatal disease in dogs and people. In our prior studies, we reported the best protect from WCAV and in the current study, we will assess various formulations of vaccine and length of protection using the best vaccine formulation; both against blood-borne infection, then test protection against tick transmission and finally against heterologous strains of the pathogen. The 4th project investigates pathogenesis of an important foreign animal tick-borne disease of ruminants; the heartwater disease caused by Ehrlichia ruminantium. This study investigates the risk of cattle from E. ruminantium by direct needle infection and from a tick native to the mainland USA, Amblyomma maculatum.

4. Scientific Description of Experimental Design

In language a scientific colleague can understand, provide a step-by-step, general description of the animal experiments you will perform including experimental groups and timing of procedures and manipulations. For complicated experimental designs, including a flow chart, diagram, or table in the Attachments section is recommended to help the ACUC understand what is proposed. DO NOT describe details of the procedures here as such details are requested later in the form.

Project 1) Active NIH grant # R01 AI070908: Vector and host contributions to the regulation of E. chaffeensis gene expression

Brief summary: Perform mutational analysis and in vivo screening to identify genes essential for the E. chaffeensis pathogenesis in vertebrate and tick hosts. We will generate large pools of E. chaffeensis transposon mutants in support of this objective. Our funding was approved to generate 200 mutant organisms. These mutants will then be screened to define the pathogenesis using the canine infection model; three experiments were proposed to accomplish this goal.

Background: The family Anaplasmataceae contains several obligate, intracellular, Gram-negative bacteria which include species of the genera Ehrlichia and Anaplasma and responsible for causing infections in dogs and people, as well as in several other vertebrate hosts. We recently performed mutational analysis and demonstrated that mutations in three different genes of E. chaffeensis caused attenuated growth of the organism in vivo (Cheng et al. 2013). These data formed the basis for our funded NIH-R01 grant application having the three specific aims. Aim 3 requires the use of animal studies, i.e., to perform mutational analysis and in vivo screening to identify additional genes essential for the E. chaffeensis pathogenesis in vertebrate and tick hosts. We have completed part the proposed experiments of this aim already at K-State as per an IACUC approval (Wang et al. 2020). This application will focus on the remaining proposed portion of the experiment. Dog is chosen as the infection model for the proposed experiments because it is an incidental host in acquiring E. chaffeensis similar to humans. Moreover, our several recent experimental studies demonstrated that this host serves as an excellent infection model, where the pathogen infection causing a very mild disease and the infection persists in (Nair et al. 2016). Our experimental infection studies demonstrated that dogs develop only mild fever (rise in only up to 1.5oC body temperature), while maintaining persistent infections with detectable hematological changes, host response and having milder histopathological changes.

Experimental plan:
Animal details. We will use about 6-month-old beagle breed dogs (representing both sexes equally) weighing approximately 8-10Kg for all of our studies. Animals will be purchased from a USDA approved vendor and acclimated for one week prior to introduction into the study. The study timeline and end points are described under each experiment.

Experiment involving animals: We proposed to screen 200 E. chaffeensis mutants in the canine host. As of now, we completed screening 60 mutants as 6 pools by infecting three dogs each with about 10 mutants in each pool. A total of 18 were used under this objective as part of the current protocol at K-State. In this protocol, we will expect to screen 14 pools (maximum) of mutants to complete the project goals. Each pool of up to 10 mutants will be used and in three independent animals (n=3) per pool which totals 42 animals. The infection status will be assessed twice a week for two months. Nymphal ticks (typically about 250) will be allowed to acquisition feed on animals starting from day 5 post infection. Tick cells (containers that hold ticks) will be placed on dogs and covered with sheep soc (made of Nylon Spandex for easy flexibility) (Sheepman Supply co. or something similar) by following the procedures similar to those done on deer, except that there is no need for anesthetize the dogs. For these experiments, the backs of the animals will be shaved with veterinary clippers. A custom designed tick containment chamber (modified top of Nalgene jar containing screw cap lid) will be glued to polyvinyl membrane with a center circular opening. The chamber will then be glued to animals with industrial adhesive (commercially available). The chambers have round bottom smooth surface and once glued, the chambers remain attached for several weeks until polyvinyl membrane is lifted off the skin with the hair growth. To ensure that the chambers are tightly attached, tick infestations will be performed only after about 24 h following the attachment of the chambers. We will monitor for the retainment of the chambers on the animals, as well as their firm attachment. If dogs attempt to remove the chambers, we will place Elizabethan neck collars to restrict grooming. The chambers will be covered with sheep sox. To perform the tick infestation, lids of the chambers will be unscrewed, ticks will be placed inside, and the chambers will then be tightly closed with the lids and animals will be covered back with sheep sox. About 7 days following tick attachment, ticks will be collected by opening the chamber lids. We will evaluate

ticks from each animal following the molting to adult stage to assess which mutants are acquired by ticks. Together, the assessments of blood (10 ml blood drawn twice a week from cephalic veins for the first two weeks and then on once a week) and tick sampling will help us determine which genomic regions of E. chaffeensis that are critical for the in vivo growth in an incidental host model with important implications in extending the observations in understanding pathogenesis in people (total dogs for this sub-experiment are 42).

Animal monitoring plan: After infection, animals will be observed twice daily with once daily monitoring the body temperatures. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.

Blood sampling and other procedures: In the experiments, animals will be kept for 60 days each to monitor the mutant E. chaffeensis circulation in blood. Blood sampling will be done twice a week from cephalic veins (10 ml each) for the first two weeks and then once a week thereafter. Total blood draws will be 11 times per animal. About 6- to 8-month-old dogs of the breed 'Beagle' will be used for these experiments. For convenience, we will either use all males or all females in each experimental group, while maintaining equal numbers of males and females throughout the study. The weight of each animal will be about 15 to 20 pounds. Diphenhydramine (Benadryl) (1mg per pound) will be orally administered to all animals about 30 minutes prior to inoculation with Ehrlichia. (The stock concentration to be used is 2.5 mg/ml; 6 to 8 ml per animal or 15-to-20-pound dogs.) Benadryl is administered to prevent any possible anaphylactic shock resulting from injection of organisms containing traces of serum or other animal products likely present in the culture media.

At the end of the study: At the completion of the study, dogs will be transferred to another study or will be adopted out after a four-week treatment with doxycycline. This infection is very common in dogs and pose milder disease and so it will not be a concern to either the dogs or to pet owners. The infection with E. chaffeensis is very common in dogs and poses milder disease (Bowman et al., 2009 and Beall et al. 2012). It will not be a concern to either the dogs or to pet owners. Thus, subjecting to adaptation or transferring to other research projects are fully justified. These animals will be transferred to other projects within the university as per the needs of a project(s) or may also be opened up for the adaption if such option is not available.

References:

Cheng C, Nair ADS, Indukuri VV, Gong S, Felsheim RF, Jaworski D, Munderloh UG and Ganta RR. Targeted and random mutagenesis of Ehrlichia chaffeensis for the identification of genes required for in vivo infection. PLoS Pathog. 2013 Feb;9(2):e1003171. doi: 10.1371/journal.ppat.1003171. Epub 2013 Feb 14.

Wang Y, Nair ADS, Alhassan A, Jaworski DC, Liu H, Trinkl K, Hove P, Ganta CK, Burkhardt N, Munderloh UG and Ganta RR. Multiple Ehrlichia chaffeensis genes critical for its persistent infection in a vertebrate host are identified by random mutagenesis coupled with in vivo infection assessment. Infect Immun. (2020) 88(10) DOI: 10.1128/IAI.00316-20.

Nair AD, Cheng C, Ganta CK, Sanderson MW, Alleman AR, Munderloh UG, Ganta RR. Comparative experimental infection study in dogs with Ehrlichia canis, E. chaffeensis, Anaplasma platys and A. phagocytophilum. PLoS One. 2016 Feb 3;11(2):e0148239. doi: 10.1371/journal.pone.0148239

Bowman, D., Little, S. E., Lorentzen, L., Shields, J., Sullivan, M. P., & Carlin, E. P. (2009). Prevalence and geographic distribution of Dirofilaria immitis, Borrelia burgdorferi, Ehrlichia canis, and Anaplasma phagocytophilum in dogs in the United States: results of a national clinic-based serologic survey. Vet

Parasitol, 160(1-2), 138-148.

Beall MJ, Alleman AR, Breitschwerdt EB, Cohn LA, Couto CG, Dryden MW, Guptill LC, Iazbik C, Kania SA, Lathan P, Little SE, Roy A, Sayler KA, Stillman BA, Welles EG, Wolfson W, Yabsley MJ. Seroprevalence of Ehrlichia canis, Ehrlichia chaffeensis and Ehrlichia ewingii in dogs in North America. Parasit Vectors. 2012 Feb 8;5:29. doi: 10.1186/1756-3305-5-29. doi:10.1186/1756-3305-5-29

Project 2) Active NIH grant # R01 AI152418: Vaccines Against Ehrlichia and Anaplasma Species Infections

Brief summary: Tick-borne pathogens belong to the genera Ehrlichia and Anaplasma continue to emerge as a major public health concern during the last 3-4 decades. They include the emerging diseases; human monocytic ehrlichiosis, human ewingii ehrlichiosis, and human granulocytic anaplasmosis caused by Ehrlichia chaffeensis, Ehrlichia ewingii, and Anaplasma phagocytophilum. We recently reported the development of a modified live attenuated vaccine (MLAV) inactivating an important gene (ECH_0660) against E. chaffeensis that conferred protection against infection challenge from blood transfusion and from infected ticks (Nair et al. 2015 and McGill et al. 2016). Goals of this funded project are 1) to evaluate the duration of protection offered from E. chaffeensis MLAV against wild type infection challenge through blood stream and tick transmission; 2) to determine if immunity to the vaccine protects against genetically E. chaffeensis strains; and 3) to evaluate similar MLAV from related Ehrlichia and Anaplasma to protect against infections.

Background: Rickettsial diseases caused by pathogens of the Anaplasmataceae family, including members of the genera Ehrlichia and Anaplasma, are responsible for frequent infections in people over the past three decades and are a leading cause of tick-borne infections in humans throughout the USA and many parts of the world. These pathogens also infect diverse vertebrate hosts, although also are causing a milder disease in majority of host species. These pathogens have evolved strategies to evade host immunity and cause persistent infections. Through our recently established mutagenesis experiments, we created E. chaffeensis mutants that contained insertions causing functional gene disruptions. An insertion mutation in the ECH_0660 gene resulted in the pathogen's rapid clearance from two vertebrate hosts (Cheng et al. 2013). Vaccination with this mutant induced a strong host response and offered complete protection against blood stream and tick transmission infection with wild-type E. chaffeensis one month after vaccination (Nair et al. 2015 and McGill et al. 2016). Previously, we performed molecular characterization of several E. chaffeensis isolates and reported that the isolates represent three distinct genetic groups (Cheng et al. 2003). We proposed the following three specific aims (all three involves the use of animals): 1) Evaluate the duration of immunity offered by the ECH_0660 gene mutant live attenuated vaccine (MLAV) against wild type infection challenge through blood stream and tick-transmission. 2) Evaluate the protection of the MLAV against genetically distinct E. chaffeensis strains. 3) Evaluate mutants in related Ehrlichia and Anaplasma species for their efficacy as live attenuated vaccines in conferring protection against the pathogens' infection into blood stream and by tick-transmission. As part of the completed research during the last two years, we completed the goals of aim 1, thus, we propose in executing experiments planned as part of aims 2 and 3 which we call as experiments 1 and 2.

Experimental plan:

Animal details. We will use about 6-month-old beagle breed dogs (representing both sexes equally) weighing approximately 8-10Kg for all of our studies. Animals will be purchased from a USDA approved vendor and acclimated for one week prior to introduction into the study. The study timeline and end points are described under each experiment.

Experiment 1: Evaluation of cross protection induced by MLAV against different E. chaffeensis strains
Experiment 1a) Comparison of Arkansas isolate-derived MLAV protection against St. Vincent and Jax infection challenges by I.V. and tick-transmitted infection

This experiment will have 8 groups (n=6); groups 1-4 will receive the Arkansas isolate derived MLAV intravenously, while Groups 5-8 will serve as infection controls. Groups 1 and 2 will receive I.V. infection challenge one month after vaccination with wild type St. Vincent and Jax culture infection challenges, respectively. As per our prior published data, infection challenge following one month of vaccination with attenuated mutant induce sufficient host immune response in offering complete protection against blood stream infection and tick transmission challenges with wild-type E. chaffeensis (1, 2). Groups 3 and 4 will be similar to Groups 1 and 2, except that the infection challenges will be performed by tick-transmission. Groups 5 and 6 (n=6) will serve as non-vaccinated controls but will be challenged via I.V. and Groups 7 and 8 (n=6) transmitted will be challenged tick- transmitted challenge with the St. Vincent or Jax isolates, respectively similar to groups 1-4 above.

Table 1. Experimental design to test Arkansas isolate-derived MLAV protection against St. Vincent and Jax infection challenges by I.V. and tick-transmitted infection
Group Vaccine # of animals* Infection challenge.
1 Arkansas MLAV 1X I.V. 6 (3F+3M) I.V.E. chaffeensis (St. Vincent)
2 Arkansas MLAV 1X I.V. 6 (3F+3M) I.V.E. chaffeensis (Jax)
3 Arkansas MLAV 1X I.V. 6 (3F+3M) tick transmission E. chaffeensis (St. Vincent)
4 Arkansas MLAV 1X I.V. 6 (3F+3M) tick transmission E. chaffeensis (Jax)
5 Infection Control 6 (3F+3M) I.V.E. chaffeensis (St. Vincent)
6 Infection Control 6 (3F+3M) I.V.E. chaffeensis (Jax)
7 Infection Control 6 (3F+3M) tick transmission E. chaffeensis (St. Vincent)
8 Infection Control 6 (3F+3M) tick transmission E. chaffeensis (Jax)

*48 animals

Experiment 1b) Comparison of the St. Vincent isolate-derived MLAV protection against Arkansas and Jax infection challenge by I.V. infection and by tick-transmission.
In this experiment, the St. Vincent isolate mutant MLAV will be used as the vaccine, and infection challenges will be performed with wild type I.V. infection and tick transmission with Arkansas and Jax isolates of E. chaffeensis. This experiment will have 4 vaccinated groups (n=6) and four non-vaccinated groups (n=6); groups 1-4 will receive the St. Vincent isolate derived MLAV intravenously, while Groups 5-8 will serve as infection controls. Infection challenges will be performed with wild type I.V. infection and tick transmission with Arkansas and Jax isolates. Groups 1 and 2 will receive I.V. infection challenge one month after vaccination with wild type Arkansas and Jax culture infection challenges, respectively. Groups 3 and 4 will be similar to Groups 1 and 2, except that the infection challenges will be performed by tick-transmission. Groups 5 and 6 (n=3) will serve as non-vaccinated controls and will receive I.V. infection challenge with the Arkansas or Jax isolate cultures, respectively. Groups 7 and 8 (n=3) will be challenged via tick-transmitted challenge with the Arkansas or Jax isolate infected ticks. Since we have sufficient number of control animals in the previous experiments, we reduced the number of control animals (n=3) in this study.

Table 2. Experimental design to test St. Vincent isolate-derived MLAV protection against Arkansas and Jax isolates infection challenges by I.V. and tick-transmitted infection
Group Vaccine # of animals* Infection challenge.
1. St. Vincent MLAV 1X I.V. 6 (3F+3M) I.V.E. chaffeensis (Arkansas)
2. St. Vincent MLAV 1X I.V. 6 (3F+3M) I.V.E. chaffeensis (Jax)
3. St. Vincent MLAV 1X I.V. 6 (3F+3M) tick transmission E. chaffeensis (Arkansas)
4. St. Vincent MLAV 1X I.V. 6 (3F+3M) tick transmission E. chaffeensis (Jax)
5. Infection Control 3 (F or M) I.V.E. chaffeensis (Arkansas)
6. Infection Control 3 (F or M) I.V.E. chaffeensis (Jax)
7. Infection Control 3 (F or M) tick transmission E. chaffeensis (Arkansas)
8. Infection Control 3 (F or M) tick transmission E. chaffeensis (Jax)

*36 animals

Experiment 1c) Comparison of the Jax isolate-derived MLAV protection against Arkansas and St. Vincent infection challenge by I.V. infection and by tick-transmission.
In this experiment, the Jax isolate mutant MLAV will be used as the vaccine, and infection challenges will be performed with wild type I.V. infection and tick transmission with Arkansas and St. Vincents isolates. This sub-aim will have four vaccinated groups (n=6) and all four groups will receive the MLAV and will also include four non-vaccinated control groups (n=3). Groups 1 and 2 will receive I.V. infection challenge one month after vaccination with wild type Arkansas and St. Vincents culture infection challenges, respectively. Groups 3 and 4 will be similar to Groups 1 and 2, except that the infection challenges will be performed by tick-transmission with the respective isolate infections. Groups 5 and 6 (n=3) will serve as non-vaccinated controls challenged via I.V. and Groups 7 and 8 (n=3) will be challenged via tick-transmitted challenge with Arkansas or St. Vincent isolates, respectively, similar to groups 3 and 4.

Table 3. Experimental design to test Jax isolate-derived MLAV protection against Arkansas and St. Vincent isolates infection challenges by I.V. and tick-transmitted infection

Group Vaccine # of animals Infection challenge .
1. Jax MLAV 1X I.V. 6 (3F 3M) I.V.E. chaffeensis (Arkansas)
2. Jax MLAV 1X I.V. 6 (3F 3M) I.V.E. chaffeensis (St. Vincent)
3. Jax MLAV 1X I.V. 6 (3F 3M) tick transmission E. chaffeensis (Arkansas)
4. Jax MLAV 1X I.V. 6 (3F 3M) tick transmission E. chaffeensis (St. Vincent)
5. Infection Control 3 (F or M) I.V.E. chaffeensis (Arkansas)
6. Infection Control 3 (F or M) I.V.E. chaffeensis (St. Vincent)
7. Infection Control 3 (F or M) tick transmission E. chaffeensis (Arkansas)
8. Infection Control 3 (F or M) tick transmission E. chaffeensis (St. Vincent)

*36 animals

Experiment 2) Evaluation of related Ehrlichia and Anaplasma species MLAV for their efficacy in conferring protection against wild type infection in the blood stream and by tick-transmission.
Experiment 2a): Evaluation of Ecaj_0381 disrupted MLAV's ability to confer protection against E. canis infection by I.V. into blood stream and by tick transmission.
This study will have two vaccination groups (n=6) and both groups will receive the same E. canis MLAV. Group 1 will receive I.V. infection challenge, while Group 2 will receive tick-transmission infection one month after vaccination. Groups 3 and 4 (n=3) will serve as non-vaccinated controls, which will receive infection challenges similar to Groups 1 and 2. For the control groups also we will use n=6.

Table 4. Experimental design to test E. canis MLAV protection against E. canis infection challenges by I.V. and tick-transmitted infection

Group Vaccine # of animals* Infection challenge.
1. E. canis MLAV 1X I.V. 6 (3F 3M) I.V.E. canis (wild type)
2. E. canis MLAV 1X I.V 6 (3F 3M) tick transmission E. canis (wild type)
3. Infection Control 6 (M or F) I.V.E. canis (wild type)
4. Infection Control 6 (M or F) tick transmission I.V.E. canis (wild type)

*24 animals

Experiment 2b): Evaluation of Aph_0634 disrupted MLAV's ability to confer protection against A. phagocytophilum infection challenge by I.V. infection into blood stream and by tick transmission.

In this study, A. phagocytophilum Aph_0634 mutant MLAV will be used as the vaccine similar to the previous

experiment. Infection challenges will be performed with a human isolate of A. phagocytophilum (HGA2) using the wild type cultured organisms for I.V. infection and using infected ticks. As in the previous experiment, this study will include two vaccination groups (n=6) and two non-vaccinated control groups (n=6).

Table 5. Experimental design to test A. phagocytophilum MLAV protection against A. phagocytophilum infection challenges by I.V. and tick-transmitted infection

Group Vaccine # of animals* Infection challenge .
1. A. phagocytophilum MLAV 1X I.V. 6 (3F 3M) I.V.A. phagocytophilum (wild type)
2. A. phagocytophilum MLAV 1X I.V 6 (3F 3M) tick transmission A. phagocytophilum (wild type)
3. Infection Control 6 (M or F) I.V.A. phagocytophilum (wild type)
4. Infection Control 6 (F or M) tick transmission A. phagocytophilum (wild type)

*24 animals

Mutant Live Attenuated Vaccines (MLAVs): The MLAVs contain either modified E. chaffeensis, E. canis or A. phagocytophilum in vitro cultured mutant organisms washed with PBS and resuspended in PBS at a dose rate of 2X108 organisms/mL. Vaccines will be administered as I.V. (1 mL/animal).

Infection challenge dose: Infection challenges will be performed with 2X108 bacteria grown in appropriate cell culture by I.V. inoculation method; we chose this dose as we previously reported in an infection model utilizing this dose (Nair et al., 2016). E. canis organisms will be quantified in the culture; the culture will be centrifuged to concentrate and remove the culture media and resuspended into 1x PBS to a final concentration of 2X108 bacteria per 1 ml for use in inoculation experiments.

Intravenous injections: Each dog will receive 1 ml of the inocula into left or right cephalic vein using a 23 G butterfly needle. The vaccination site will be aseptically prepared by shaving hair (approximately 2cm2) and cleaning with 70% ethanol. To prevent any possibility of developing anaphylactic reactions, Benadryl (diphenhydramine) will be administered 30 min prior to any intravenous vaccine or challenge inoculum administration.

Tick transmission challenge: Infection challenge with tick transmission will be done as per our published protocol. Twenty-five adult infected tick pairs (25 males and 25 females) will be allowed to transmission feed on vaccinated dogs for 7 days. Engorged nymphs (obtained from a commercially available source) will be infected with E. chaffeensis, E. canis or A. phagocytophilum by needle inoculation and allowed to molt to the adult stage (Cheng et al. 2015 and Jaworski et al., 2016). To prepare for a tick transmission experiment, we will prepare a tick containment cell for each dog. In our system, we will use containment chambers constructed from the tops of Nalgene jars that are each fitted with a screen and polyvinyl gasket that will be directly glued (3M Scotch-Weld 4799 adhesive) to the shorn back of a dog. Dogs are manually held for the application of the tick containment cell. The shaved area will be approximately 4 inches in diameter and to either the right or left side of the dog over the midback area. The placement of containers will be done 24 hours prior to tick infestation. In addition, the dogs will be fitted with a collar to restrict grooming near the containment chamber. Tick infestations will be accomplished by placing 25 female and 25 male ticks on each dog. We will count ticks to be used for each dog carefully. The transfer of ticks to dogs will be performed by unscrewing the screened top of the container and placing the ticks on the dog. The top of the chamber will be re-secured immediately, and dogs will be returned to individual housing. The dog will be restricted from group play during the 7-day period that the tick containment cells are present. Dogs, tick containment chambers and tick attachments will be monitored daily until all ticks are removed from dogs. Extreme care will be taken, and all ticks will be counted (live or dead) when partially fed ticks are removed on day 7. The Nalgene top of the container will be removed from the polyvinyl gasket and the gasket will be removed by shaving. The dogs will be monitored for an additional four weeks.

Animal monitoring plan: After infection with live vaccines and after infection challenges, animals will be

observed twice daily with once daily monitoring the body temperatures. Body weights will be measured twice a week. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.

Blood sampling: All blood collections will be done from jugular, or anterior cephalic or lateral saphenous veins using 20 or 22 gauge needles.

Vaccination phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week during vaccination phase. In addition, 1 ml of whole blood in EDTA tube will also be obtained for performing CBC analysis (once a week) for one month following vaccination. One ml of blood in EDTA tube will also be collected and used for checking the infection status twice a week for the first month. In experiment 1, after the first month of vaccination, about 20 ml blood will be collected once in every two weeks until challenge.

Challenge phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week until end point. In addition, 2 ml blood in EDTA tubes will be collected twice a week for assessing the systemic bacterial load and 1ml blood will be collected for CBC analysis. If dogs exhibit high fever or other clinical symptoms, additional 1 ml blood may be collected a third time in a week to monitor the infection status.

At the end of the study: At the completion of the study, dogs will be transferred to another study or will be adopted out after a four-week treatment with doxycycline. The infections with E. chaffeensis, E. canis and A. phagocytophilum are very common in dogs and pose milder disease and will not be a concern to either the dogs or to pet owners. Thus, subjecting to adaptation or transferring to other research projects are fully justified.

References:
Nair ADS, Cheng C, Jaworski DC, Ganta S, Sanderson MW, Ganta RR: Attenuated Mutants of Ehrlichia chaffeensis Induce Protection against Wild-Type Infection Challenge in the Reservoir Host and in an Incidental Host. Infection and immunity 2015, 83(7):2827-2835.

McGill JL, Nair ADS, Cheng C, Rusk RA, Jaworski DC, Ganta RR: Vaccination with an Attenuated Mutant of Ehrlichia chaffeensis Induces Pathogen-Specific CD4+ T Cell Immunity and Protection from Tick-Transmitted Wild-Type Challenge in the Canine Host. PLoS One 2016, 11(2):e0148229.

Cheng C, Nair ADS, Indukuri VV, Gong S, Felsheim RF, Jaworski D, Munderloh UG and Ganta RR. Targeted and random mutagenesis of Ehrlichia chaffeensis for the identification of genes required for in vivo infection. PLoS Pathog. 2013 Feb;9(2):e1003171. doi: 10.1371/journal.ppat.1003171. Epub 2013 Feb 14.

Cheng C, Paddock CD, Reddy Ganta R: Molecular heterogeneity of Ehrlichia chaffeensis isolates determined by sequence analysis of the 28-kilodalton outer membrane protein genes and other regions of the genome. Infection and immunity 2003, 71(1):187-195.

Project 3) Active NIH grant # R01 AI152417: Rocky Mountain Spotted fever vaccine development

Brief summary: Rocky Mountain spotted fever remains a life-threatening tick-borne disease of people and continues to be a public health concern in the USA and several North, Central and South American countries.

During the last two decades, reported RMSF cases continue to rise in parts of North America. This NIH funded application investigates RMSF vaccine development using a relevant animal-tick-pathogen infection model (dog and tick). At the completion of the project, we expect to have a fully developed vaccine useful in devising strategies to control the disease.

Background: Tick-transmitted rickettsial diseases of the genera Anaplasma, Ehrlichia, and Rickettsia remain a growing public health concern in the USA and many parts of the world. The diseases include one of the oldest known rickettsial diseases, Rocky Mountain spotted fever (RMSF) caused by Rickettsia rickettsii. RMSF remains a serious disease of people and dogs for about a century and continues to be a public health concern in the USA and several North, Central and South American countries resulting from a tick bite (Alvarez-Hernandez et al., 2017; Piranda et al. 2008; Labruna et al., 2009; Piranda et al., 2011; Drexler et al., 2017; Hatcher et al. 2018; Londono et al. 2019;) [4, 7-19]. Clinical signs of RMSF include fever, headache, nausea, vomiting, muscle pain, lack of appetite, and rash. The disease can progress rapidly to a life-threatening illness in untreated patients, resulting in high mortality rates ranging from 30-80% [4, 20]. During the last two decades, reported RMSF cases continue rising in parts of North America (Drexler et al. 2017; Tinoco-Gracia et al. 2018). Since dogs develop disease similar to people, a vaccine to prevent the disease in this host will most likely be effective in controlling the disease spread from wildlife, ticks and also infections from dogs to people. We recently demonstrated that whole cell inactivated antigens of R. rickettsii offer complete protection against virulent infection challenge in the canine host (Alhassan et al.; 2019). Our prior published work offers the strongest justification for the proposed detailed investigation for which we received NIH grant funding. The following are the proposed objectives.

1) Evaluate inactivation methods for preparing WCA-S (Sheila Smith strain) and adjuvants in defining the vaccine protection.
2) Evaluate the duration of immunity
3) Evaluate protection against tick-transmitted challenges.
4) Evaluate WCA protection against R. rickettsii heterologous strain infection challenges.

Experimental plan:
Three different inactivation methods will be used to prepare WCA-S (whole cell inactivated antigen from Sheila Smith strain); heat, formalin and hydrogen peroxide.

Animal details: Purpose bred beagle dogs (4-6 months old of both sexes), weighing approximately 8-10 kg, obtained from a Class A USDA vendor, will be housed in indoor climate-controlled facilities with ad libitum food and water and adequate spacing to allow regular exercise activities. They will be acclimated for one week prior to introduction into the study. The study timeline and end points are described under each experiment.

Experiment 1: Evaluate inactivation methods for preparing WCA-S and adjuvants in defining the vaccine protection.

Vaccine assessments with WCA prepared by three different inactivation methods and using three different adjuvants: In our recent study, we used 70 µg of heat inactivated whole cell antigens of R. rickettsii Shelia Smith strain diluted in PBS with final concentration of 2.5% Montanide™ Gel.This experiment will be performed similarly; 9 vaccination groups will be included (n=6 for each group; 3 males and 3 females). One group will receive only adjuvant (n=6 and two animals each per adjuvant) and then will be subjected to infection challenge to serve as infection controls. (Total number animals for this experiment will be 60.)

We will not include uninfected controls as we have ample data generated previously using such controls. Vaccines prepared with three inactivation methods (heat, formaldehyde and H2O2) and with three different adjuvants (Montanide gel, QS-21 saponin and Aluminum hydroxide) will be used in this experiment. Similarly, adjuvant only preparations will be administered to control groups. The vaccination protocol will be similar to our recent publication with a priming vaccination on day 0, booster vaccination on day 21 and I.V. infection

challenge with 105 R. rickettsii Shelia Smith strain organisms recovered from embryonated chicken eggs on day ~50 (Alhassan et al., 2019). Infection progression will be monitored for 30 days. All dogs in all groups will be monitored daily for health, clinical and behavioral changes, and twice weekly for hematological changes by complete blood count analysis. Body weights will be measured once a week. Body temperatures will be measured twice a week during the vaccination phase and daily following infection challenges. Temperature assessments will be done at similar times each day. Blood sampling will be performed as per the description in our recent publication for CBC analysis, to evaluate T- and B-cell responses, and to monitor bacterial burden of circulating R. rickettsii. At the end of the experiment, the animals will be euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) using a commercial euthanasia solution. A full necropsy will be performed, and tissue samples will be assessed for gross pathology and histopathology, as in (Alhassan et al., 2019). While our preference is to do all the groups at one time, we will be able to do this experiment in two phases if we are limited by the constraints of the facilities available for housing. (Note: depending on the resource availability and personnel management, we may opt to perform this experiment as two parts.)

Note: A minor modification will be submitted prior to experiment 1 to provide the exact details of which formulations are to be used once the results have been obtained.

Table 1.
Group Vaccine vaccination date* # of animals **. Infection Challenge***
1. (Heat & Montanide gel) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
2. (Heat & QR-21 saponin) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
3. (Heat & Aluminum hydroxide) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
4. (Formaldehyde & Montanide gel). Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
5. (Formaldehyde & QR-21 saponin). Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
6. (Formaldehyde & Aluminum hydroxide) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
7. (H2O2 & Montanide gel) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
8. (H2O2 & QR-21 saponin) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
9. (H2O2 & Aluminum hydroxide) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
10. Infection control (2 per adjuvant) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii

*All vaccinations will be performed subcutaneously.
**60 animals
**The infection challenge will be performed for all 10 groups with Shelia Smith strain of R. rickettsii.

Experiment 2: Assess the duration of immunity of WCA-S prepared using the optimum vaccine formulation.

In this experiment, we will investigate the duration of immunity induced by WCA-S. We will select the best vaccine formulation (inactivation method and adjuvant) as per the results identifying the most efficacious in Experiment 1. The criteria for selecting the best vaccine formulation will be based on the data assessments comparing the protection in clearing the clinical disease coupled with immune response determined by comparing the results of three different vaccine preparations and adjuvants. All data will be assessed and discussed by our research team to reach this conclusion. If all three vaccines will yield similar results, then we will add economic costs to determine our chose for the next set of experiments.

Note: A minor modification will be submitted after the completion of Experiment 1, and prior to any remaining experiments, to provide which vaccine formulation will be used for the experiments.

We selected four time points for assessing the protection following the booster vaccination: 2, 4, 8 and 12 months. This experiment will include four groups (n=6) and a control group (n=4) to serve as non- vaccinated infection challenge group for comparing the protection. The reason that n=4 will be sufficient to serve in the control group as by this point we will have sufficient knowledge regarding RMSF in the dog model, which will be based on our prior work as well as the results generated from our previous experiment. (Total animals for

this experiment will be 28.) Vaccination protocol will be followed as in the previous experiment. Similarly, all assays to assess the bacterial clearance, host immune responses, hematological parameters and pathological assessments will be followed as per the previous experiment, except that the infection challenge times will be different for each group. Peripheral blood and sera will be collected from the animals from all groups immediately prior to each challenge, as well as on different days post R. rickettsii challenge to evaluate cellular and humoral memory responses throughout the course of the study

Table 2.
Group Vaccine. Vaccine* days. # of animals** Infection challenge*** .
1. Vaccine formulation Day 0 and 21 6 (3F+3M) 12 months after vaccination; I.V. 105 R. rickettsii
2. Vaccine formulation Day 0 and 21 6 (3F+3M). 8 months after vaccination; I.V. 105 R. rickettsii
3. Vaccine formulation Day 0 and 21 6 (3F+3M). 4 months after vaccination; I.V. 105 R. rickettsii
4. Vaccine formulation. Day 0 and 21 6 (3F+3M). 2 months after vaccination; I.V. 105 R. rickettsii
5. Infection Control (no vaccination) 4 (2F+ 2M) infection with groups 1-4; I.V.105 R. rickettsii

*All vaccinations will be performed subcutaneously.
**28 animals
***The infection challenge will be performed with the Shelia Smith strain of R. rickettsii.

Experiment 3: Evaluate protection against tick-transmitted challenges.

In this experiment, we will investigate the efficacy of the WCA-S vaccine against tick-transmitted challenge with R. rickettsii Sheila Smith strain. We will use the optimized vaccine formulation (with inactivation and adjuvant formulation) for this experiment. Three groups of dogs will be used in the tick- transmission challenge experiments. Two groups will be used for tick transmission challenge (n=6), while the third group will be used for I.V. infection challenge. We will reduce the number of dogs to 4 in the 3rd group, as we anticipate having sufficient data already in place regarding the efficacy of the WCA vaccine against I.V. infection challenge. (Total number of animals for this experiment will be 12.) The 1st and 3rd group will receive WCA primary and booster vaccinations as described above. The 2nd group will serve as the non-vaccinated and tick-transmission infection control group. Infection challenge will be performed one month after the final WCA immunization, or as per the optimum time point established in our time course experiment described above. Dogs in groups 1 and 2 will receive tick transmitted infection challenge by allowing 25 pairs of R. rickettsii-infected adult D. variabilis ticks to feed on the dogs for a week. The third group will receive an I.V. infection challenge with 105 R. rickettsia organisms. All assays to assess the vaccine protection will be similar as in the previous experiments.

We will use engorged D. variabilis nymphal ticks (within 24 - 48 h post blood meal) obtained from a commercial vendor {we typically use BEI Resources (Manassas, VA) and the Tick Rearing Facility of Oklahoma State University (Stillwater, OK)} to inject with chicken egg embryo-derived R. rickettsii organisms suspended in PBS at a concentration of 100 bacteria per micro liter. Needle puncture inoculation (with 26-gauge needle) will be placed into the ventral side of the ticks. Ticks will then be allowed to molt to adult stage at room temperature by exposure to 14 h light and 10 h dark cycle in a 96% humidity chamber [118]; we followed this protocol as part of several earlier studies. About 10 randomly selected ticks will be assessed for the infection rates using individually isolated genomic DNAs as templates for the nested PCR targeting to AdR2 gene of R. rickettsii [22]. This method, however, may not yield infected ticks and is the reason we proposed experiments in this application seeking approval to generate infected ticks following acquisition feeding on R. ricketsii-infected dogs (described in the experimental section).
Table 3.
Group Vaccine* # of animals** Infection challenge with Sheila Smith strain***
1. WCA vaccine; 0 and 21 days 6 (3F+3M) after 1 month; tick transmission R. rickettsii
2. Infection controls. 6 (3F+3M). tick transmission R. rickettsii
3. WCA vaccine; 0 and 21 days. 4 (2F+2M) after 1 month; infection by I.V.105 R. rickettsii

*All vaccinations will be performed subcutaneously.
**16 animals
**The infection challenge will be performed with the Shelia Smith strain of R. rickettsii.

Experiment 4: Evaluate WCA protection against R. rickettsii heterologous strain infection challenges.

Experiment 4.1: Compare Sheila Smith strain derived WCA protection against Morgan strain infection challenge. In this experiment, WCA will be prepared using the Sheila Smith strain R. rickettsii and primary and booster vaccinations will be performed as per previous experiment. The infection challenge will then be performed using the heterologous, virulent R. rickettsii Morgan strain by I.V. infection and tick transmission. This experiment will include four groups (n=6); Groups 1 and 2 will be vaccinated and Groups 3 and 4 will serve as non-vaccinated controls. Groups 1 and 3 will be challenged via I.V. infection with 105 R. rickettsii Morgan strain organisms, while Groups 2 and 4 will be challenged via tick-transmission using R. rickettsii Morgan strain infected D. variabilis. (Total number of animals for this experiment will be 24.) Infected ticks will be generated as outlined previously. All parameters to assess the bacterial clearance, host immune responses, hematological responses and pathological assessments will be performed as described under aim 1.

Table 4.1.
Group Vaccine* # of animals** Infection Challenge
1. Sheila Smith WCA vaccine. 6 (3F+3M) I.V.105 R. rickettsii (Morgan)
2. Sheila Smith WCA vaccine. 6 (3F+3M) tick transmission R. rickettsii (Morgan)
3. Infection Control. 6 (3F+3M) I.V.105 R. rickettsii (Morgan)
4. Infection Control. 6 (3F+3M) tick transmission R. rickettsii (Morgan)

*All vaccinations will be performed subcutaneously.
**24 animals

Experiment 4.2: Compare Morgan strain-derived WCA protection against Sheila Smith strain infection challenge.
Approach: All proposed experiments in this sub-aim will be similar to the previous sub-aim, except that we will use the Morgan strain to prepare the WCA vaccine, and dogs will be challenged with the Sheila Smith strain of R. rickettsii. For groups 3 and 4, we will use only 4 animals each, as we expect to have sufficient data related to this kind of controls. (Total number of animals for this experiment will be 20.)

Table 4.2.
Group Vaccine* # of animals**. Infection Challenge
1. Morgan WCA vaccine 6 (3F+3M) I.V.105 R. rickettsii (Sheila Smith)
2. Morgan WCA vaccine 6 (3F+3M) tick transmission R. rickettsii (Sheila Smith)
3. Infection Control 4 (2F+2M) I.V.105 R. rickettsii (Sheila Smith)
4. Infection Control 4 (2F+2M) tick transmission R. rickettsii (Sheila Smith)

*All vaccinations will be performed subcutaneously.
**20 animals

Experiment 4.3: Compare Iowa strain derived WCA protection against Sheila Smith and Morgan strains' infection challenges.
Approach: The experimental design and assessments to monitor vaccine protection will also be similar to the previous two experiments. Here, we will use Iowa strain for preparing the WCA. The experiment will include 8 groups; four groups will receive the Iowa strain WCA vaccine, and four groups will serve as non-vaccinated controls. For the four non-vaccinated control groups, we will have four animals each. We believe that n=4 will be sufficient for non-vaccinated infection control groups as we will have ample data from similar controls from previous two sub aims. Vaccinated groups will have 6 dogs each. (Total number of animals for this

experiment will be 40.) Two groups will receive the Morgan strain infection via I.V. or by tick-transmission; the remaining two groups will be challenged with the Sheila Smith strain via I.V. or tick-transmission.

Table 4.3
Group Vaccine* # of animals** Infection Challenge
1. Iowa WCA vaccine 6 (3F+3M) I.V.105 R. rickettsii (Morgan)
2. Iowa WCA vaccine 6 (3F+3M) tick transmission R. rickettsii (Morgan)
3. Iowa WCA vaccine 6 (3F+3M) I.V.105 R. rickettsii (Sheila Smith)
4. Iowa WCA vaccine 6 (3F 3M) tick transmission R. rickettsii (Sheila Smith)
5. Infection Control 4 (2F + 2M) I.V.105 R. rickettsii (Morgan)
6. Infection Control 4 (2F + 2M) tick transmission R. rickettsii (Morgan)
7. Infection Control 4 (2F + 2M) I.V.105 R. rickettsii (Sheila Smith)
8. Infection Control 4 (2F + 2M) tick transmission R. rickettsii (Sheila Smith)

*All vaccinations will be performed subcutaneously.
**40 animals

Subcutaneous injections: Dogs receiving WCA vaccines in all the above outlined experiments will be administered subcutaneously. Total of 70 micro grams of antigen will be mixed with an adjuvant in a final volume of 500 micro liters (0.5 ml) and the entire vaccine will be administered once and at one site at the back of an on animal after shaving the inoculation site.

Infection challenges: Each dog will receive 1 ml the inoculum into left or right cephalic vein using a 23 G butterfly needle. The infection site will be aseptically prepared by shaving hair (approximately 2cm x 2cm) and cleaning with 70% ethanol. To prevent any possibility of developing anaphylactic reactions, Benadryl (diphenhydramine) will be administered 30 min prior to any intravenous vaccine or challenge inoculum administration.

Tick transmission challenge: Infection challenge with tick transmission will be done as per our published protocol. Twenty-five adult tick pairs (25 males and 25 females) infected with Sheila Smith strain or Morgan strain (as per the experiments outlined above) will be allowed to transmission feed on vaccinated dogs for 7 days. To prepare for a tick transmission experiment, we will prepare a tick containment cell for each dog. In our system, we will use containment chambers constructed from the tops of Nalgene jars that are each fitted with a screen and polyvinyl gasket that will be directly glued (3M Scotch-Weld 4799 adhesive) to the shorn back of a dog. Dogs are manually restrained for the application of the tick containment cell. The shaved area will be approximately 4 inches in diameter and to either the right or left side of the dog over the mid back area. The placement of containers will be done 24 hours prior to tick infestation. In addition, the dogs will be fitted with a collar to restrict grooming near the containment chamber. Tick infestations will be accomplished by placing 25 female and 25 male ticks on each dog. We will count ticks to be used for each dog carefully. The transfer of ticks to dogs will be performed by unscrewing the screened top of the container and placing the ticks on the dog. The top of the chamber will be re- secured immediately, and dogs will be returned to individual housing. The dog will be restricted from group play during the 7-day period that the tick containment cells are present. Dogs, tick containment chambers and tick attachments will be monitored daily until all ticks are removed from dogs. Extreme care will be taken, and all ticks will be counted (live or dead) when partially fed ticks are removed on day 7. The Nalgene top of the container will be removed from the polyvinyl gasket and the gasket will be removed by shaving. The dogs will be monitored for an additional four weeks.

Animal monitoring plan: After Rickettsia rickettsii infection with I.V. and tick transmission following vaccinations and in control groups, all animals will be monitored twice daily with once daily monitoring the body temperatures. Body weights will also be measured twice a week. While we do not anticipate serious clinical signs for the vaccinated groups, all non-vaccinated infection controls are expected to develop a severe clinical disease. Onset of signs for I.V. may occur within three days while tick transmission may take about a

week. The clinical signs will include high fever, edema, lethargy and lack of appetite. We will closely monitor the animals' health and promptly communicate with the attending veterinarian for appropriate action particularly if exhibiting depression, lethargy for more than 24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.
Blood sampling: All blood collections will be done from jugular, or anterior cephalic or lateral saphenous veins using 20-22 gauge needles.

Vaccination phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week during vaccination phase for the first 30 days and then every two weeks thereafter. In addition, 1 ml of whole blood in EDTA tube will also be obtained for performing CBC analysis once a week) for one month following vaccination. One ml of blood in EDTA tube will also be collected and used for checking the infection status twice a week for the first month. In experiment 1, after the first month of vaccination, about 20 ml blood will be collected once in every two weeks until challenge.

Challenge phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week until end point. In addition, 1 ml blood in EDTA tubes will be collected alternate days for 10 days for assessing the systemic bacterial load and 1 ml blood will be collected for CBC analysis. From day 11 to 21, blood sampled twice a week for CBC and bacterial analysis. If any dogs exhibit high fever or other clinical symptoms, additional 1 ml blood may be collected on the days of clinical signs to monitor the infection status.

Euthanasia and tissue sample collection: All dogs will be sacrificed following the assessment four-week assessment following the infection challenge. Before euthanasia, approximately 50 ml blood will be collected from vein puncture. Euthanasia will be performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA). Specifically, commercial euthanasia solution, Fatal-Plus®, of volume 0.22 ml/kg (86 mg/kg of pentobarbital) will be administered I.V. after the terminal bleed. The following tissue samples will be collected postmortem; spleen, liver, lymph nodes, lung, brain and bone marrow and they will be used for final detailed assessment of infection and gross pathology status.

References:
Alvarez-Hernandez G, Roldan JFG, Milan NSH, Lash RR, Behravesh CB, Paddock CD: Rocky Mountain spotted fever in Mexico: past, present, and future. Lancet Infect Dis 2017, 17(6):e189-e196.

Piranda EM, Faccini JL, Pinter A, Saito TB, Pacheco RC, Hagiwara MK, Labruna MB: Experimental infection of dogs with a Brazilian strain of Rickettsia rickettsii: clinical and laboratory findings. Mem Inst Oswaldo Cruz 2008, 103(7):696-701.

Labruna MB, Kamakura O, Moraes-Filho J, Horta MC, Pacheco RC: Rocky Mountain Spotted Fever in Dogs, Brazil. Emerging Infectious Diseases 2009, 15(3):458-460.

Piranda EM, Faccini JL, Pinter A, Pacheco RC, Cancado PH, Labruna MB: Experimental infection of Rhipicephalus sanguineus ticks with the bacterium Rickettsia rickettsii, using experimentally infected dogs. Vector Borne Zoonotic Dis 2011, 11(1):29-36.

Drexler NA, Yaglom H, Casal M, Fierro M, Kriner P, Murphy B, Kjemtrup A, Paddock CD: Fatal Rocky Mountain Spotted Fever along the United States-Mexico Border, 2013-2016. Emerg Infect Dis 2017, 23(10):1621-1626.

Londono AF, Arango C, Acevedo-Gutierrez LY, Paternina LE, Montes C, Ruiz I, Labruna MB, Diaz FJ, Walker DH, Rodas JD: A Cluster of Cases of Rocky Mountain Spotted Fever in an Area Of Colombia Not Known to be Endemic for This Disease. Am J Trop Med Hyg 2019, 3(10):18-1007.

Tinoco-Gracia L, Lomeli MR, Hori-Oshima S, Stephenson N, Foley J: Molecular Confirmation of Rocky Mountain Spotted Fever Epidemic Agent in Mexicali, Mexico. Emerg Infect Dis 2018, 24(9):1723-1725.

Hatcher C, Karahalios B, Badam M: Septic Shock Caused by Rocky Mountain Spotted Fever in a Suburban Texas Patient with Pet Dog Exposure: A Case Report. Am J Case Rep 2018, 19:917- 919.

Alhassan A, Liu H, McGill J, Cerezo A, Jakkula LUMR, Nair ADS, Winkley E, Olson S, Marlow D, Sahni A et al: Rickettsia rickettsii Whole-Cell Antigens Offer Protection against Rocky Mountain Spotted Fever in the Canine Host. Infection and Immunity 2019, 87(2):e00628-00618.

Project 4) Active USDA cooperative agreement grant:

Brief summary: Ehrlichia ruminantium is the disease-causing agent for an important tick-transmitted foreign animal disease, Heartwater. The goals of this project are to test if the pathogen can be transmitted by an indigenous US vector tick; Amblyomma maculatum (Gulf Coast tick). Secondly, we propose to investigate if tick feeding, and salivary gland secretions can enhance virulence of E. ruminantium in cattle.

Background: Ehrlichia ruminantium, a tick-borne rickettsial bacterium, causes Heartwater disease in ruminants resulting in a severe vascular endothelial damage throughout sub-Saharan Africa and parts of the Caribbean (Marcelino et al. 2016). Subacute and subclinical forms of the disease inflict significant morbidity, while peracute and acute forms can cause high mortalities [2]. The disease severity varies greatly depending on ruminant species, the animal breeds and their geographic origins, and also for different E. ruminantium strains (Kasari et al.2010). Nearly two centuries ago, E. ruminantium and a major tick vector, Amblyomma variegatum (the tropical bont tick, also known as the Senegalese tick) from Sub-Saharan Africa were introduced to certain Caribbean islands (Vachiéry et al. 2008). In our earlier studies, we reported the first molecular evidence to confirm the origins of E. ruminantium in the Caribbean to be from parts of northern Africa; Senegal and Sudan (Reddy et al. 1996). Despite the long presence of E. ruminantium (over two centuries) in three Caribbean islands in close proximity to each other (Guadeloupe, Antigua and Marie Galante) (Kelly et all, 2011), there is no obvious evidence of the pathogen spread and severe outbreaks (Barré et all 1995). However, the presence of the pathogen and a vector in parts of the Caribbean, coupled with the availability of potential indigenous vectors, such as Amblyomma maculatum (Gulf Coast tick), are identified as a major threat to the US ruminants. For the first time, we recently established a Heartwater research program on the mainland USA and performed the first infection study with seven different E. ruminantium strains (Nair et al. 2021). All sheep exhibited clinical signs characteristic of Heartwater disease, which included labored breathing, depression, coughing and nasal discharges. Gross pathology and histopathology observations in the animals were also consistent for Heartwater. However, the animals did not develop a severe form of disease. Specifically, we only observed subacute and subclinical disease with no progression to a fatal outcome (Nair et al. 2021). Much remains to be defined relative to the potential threat of the disease to the ruminants on the mainland USA.

The goals of this project are as follows: 1) Test if E. ruminantium can be transmitted by A. maculatum, the tick having wide distribution in southeastern parts of the USA. 2) Investigate if tick feeding and salivary gland secretions can enhance virulence of E. ruminantium in cattle.
Animals in use for all experiments: Steers of 6–12-month-old; 21 steers total will be obtained from a vendor.

Experiment 1: Determine if needle injected ticks will transmit E. ruminantium. We will generate infected ticks by following the needle infection method which we developed for other related ticks and rickettsial pathogens. We will use the infected adult A. maculatum (up to 25 pairs) for transmission experiments in one group of four steers to measure virulence. Up to 25 pairs of needle infected ticks will be allowed to feed to repletion upon each animal. Infection assessment will be followed for 60 days.

Experiment 2: Determine if saliva/salivary glands (saliva extracts) mixed with cultured E. ruminantium will enhance virulence.

2A: Uninfected A. maculatum adult ticks (n=100) will be partially fed 4-6 days and will be removed before repletion (which typically takes about 10-15 days) on an uninfected steer (n=1). Salivary extracts will be collected from the ticks. The steers will be either adopted out, transferred to another project, or sold back to a farm.

2B: We will mix saliva extracts with E. ruminantium cultured organisms for use in infection experiments in the following four groups of animals (n=4):

Group 1. Mix saliva extracts + cultured E. ruminantium (2 x 10^8 bacteria) and use it for subcutaneous inoculations (SQ).
Group 2. Cultured E. ruminantium (2 x 10^8 bacteria) using SQ inoculation alone.
Group 3. Mix saliva extracts + cultured E. ruminantium (2 x 10^8 bacteria) and use it for IV inoculation.
Group 4. Cultured E. ruminantium IV inoculation alone (2 x 10^8 bacteria). IV infections will be performed in 2 ml volume of the inocula into jugular veins or
subcutaneous injections as per animal grouping.

Sample Collection: For both experiments 1 and 2, blood samples will be collected from the jugular veins using a 20-gauge needle. Blood will be collected twice per week starting 2 days prior to the start of the experiment; 10 ml for use in monitoring CBC, culture and DNA analysis and for immunological studies. Two ml each of additional blood sampling will be done daily when animals exhibit fever and clinical signs. At the time of euthanasia, up to 100 mL will be collected from the jugular vein.

Acquisition feeding of ticks for both experiments 1 and 2: To determine if E. ruminantium can be acquired by A. maculatum, nymphs will be allowed to feed on all four groups of animals when we begin to see clinical signs or between 7 to 14 days post infection challenges. Ticks will be allowed to attach for feeding on steers (about 500 naïve nymphal ticks). Ticks will be allowed to secure complete blood meals and then allowed to molt to adult stages. Infection rates in the molted ticks will then be assessed by nested PCR analysis. During tick feeding, animals will be housed separately in pens as necessary and as per the CMG recommendation. Tick cells will be placed on steers. For these experiments, the backs of the animals will be shaved with veterinary clippers. A stockinette sleeve or hard capsule (cell) will be glued to the backs of steers. The firm attachment will be verified after about 24 h and prior to allowing ticks to feed. The cell will remain attached for several weeks. We will monitor twice daily for the retainment of the cell on the animals, as well as its firm attachment. To perform the tick infestation, ticks will be placed inside the cells and closed with the rubber bands or screw cap lid. Ticks will be collected following opening of the cell. We will evaluate ticks from each animal following the molting to adult stage to assess E. ruminantium acquisition by ticks. We will try to account for all ticks on each animal by counting live and dead ticks.

Animal housing during tick feeding: Steers will be individually housed for the tick feeding experiments. Individual housing of the pens are necessary to prvent grooming of animls attempting to remove tick cells. Animals will be allowed to return to co-housing at the completion of tick feeding experiments, i.e., upon final tick removal which will take about 7-10 days.

Animal monitoring plan: After infection, animals will be observed twice daily with once daily monitoring the body temperatures. Upon the onset of symptoms, daily collections of 2 ml blood will be initiated. An attending veterinarian will be consulted for appropriate action if the animals appear seriously ill, such as exhibiting depression, lethargy for more than24 hours, changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer, increased heart rate of respiration, or any neurological symptoms.

After infection challenge: All animals in all groups will be monitored for clinical signs, hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis for the rickettsemia. All animals will be monitored for behavioral changes and any changes in their eating patterns. Body temperature will be measured daily for first two weeks and once a week thereafter until the end point of the study. Any abnormal changes noted in animals will be discussed with the CMG-assigned veterinarian for follow up action plans.

Euthanasia and tissue sample collection: All steers will be sacrificed at the end of the study by following the captive-bolt stunning method by a certified veterinarian (possibly by a VHC clinician; to be identified). Before euthanasia, approximately 100 ml blood will be collected. Euthanasia will be performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA). The following tissue samples will be collected postmortem; spleen, liver, lymph nodes, lung, brain and bone marrow and they will be used for final detailed assessment of infection and gross pathology status.

References:
Marcelino I, Holzmuller P, Stachurski F, Rodrigues V, and Vachiéry N. Ehrlichia ruminantium: the causal agent of heartwater. Chapter 13, pages 241-280. Book title: Rickettsiales: Biology, Epidemiology, Molecular Biology and Vaccine Development, Ed. Sunil Thomas (Ed.) (2016). Springer Publishers

Kasari TR, Miller RS, James AM, Freier JE. Recognition of the threat of Ehrlichia ruminantium infection in domestic and wild ruminants in the continental United States. J Am Vet Med Assoc. 2010 Sep 1;237(5):520-30. doi: 10.2460/javma.237.5.520.

Vachiéry N, Jeffery H, Pegram R, Aprelon R, Pinarello V, Kandassamy RLY, et al. Amblyomma variegatum ticks and heartwater on three Caribbean islands. Ann N Y Acad Sci. 2008;1149:191 -5. doi:10.1196/annals.1428.081.

Reddy GR, Sulsona CR, Harrison RH, Mahan SM BM, AF B, AF' S articles by 'Barbet, AF B. Sequence heterogeneity of the major antigenic protein 1 genes from Cowdria ruminantium isolates from different geographical areas. Clin Diagnostic Lab Immunol. 1996;3:417 -22.

Kelly PJ, Lucas H, Yowell, Beati L, Dame J, Urdaz-Rodriguez J, Mahan S. Ehrlichia ruminantium in Amblyomma variegatum and domestic ruminants in the Caribbean. J Med Entomol. 2011 Mar;48(2):485-8. doi: 10.1603/me10172.

Barré N, Garris G, and Camus E. Propagation of the tick Amblyomma variegatum in the Caribbean. Rev Sci Tech. 1995 Sep;14(3):841-55. doi: 10.20506/rst.14.3.883.

Nair A., Hove P., Liu H., Wang Y, Cino-Ozuna A.G., Henningson J., Ganta C.K., and Ganta R.R. Experimental Infection of North American Sheep with Ehrlichia ruminantium. Pathogens 2021, 10, 451. https://www.mdpi.com/2076-0817/10/4/451

5. Justify

1. Justify Use of Animals in your Research

   Justify the use of animals for your experimental goals. **DO NOT** describe details of the experimental design or justify animal numbers here.

   There are no non-animal alternatives for all four proposed projects. Investigations focused on pathogenesis and vaccine development studies require the use of animals, particularly those naturally acquire infections are the best to define and develop effective methods of control.

2. Justify Animal Species

Justify the choice of species for your study.

Projects 1 and 2) Dog is the perfect animal model for such studies because it acquires E. chaffeensis, E. canis and A. phagocytophilum infections naturally like humans; both canines and humans are incidental hosts for the tick-borne diseases. Moreover tick transmission studies can be done in this animal model similar to those likely occurring naturally in this host species. Dogs develop persistent infections with all three pathogens. Clinical signs with the infections in the canine host are minor. The Beagle breed is chosen for the studies because it is the most commonly reported breed for similar studies in the literature and moreover, it is easy to work with this breed. Finally, this dog breed is commercially available for use in experimental studies.

Project 3) RMSF pathogen, Rickettsia rickettsii, causes infections in dogs and people naturally from infected Ixodid (hard) ticks. We previously demonstrated that dogs develop severe form of the RMSF in the canine host (Beagle breed) and that the WCAV confers complete protection against the infection challenge. Canine model is an ideal host for defining various aspects, including assessing host-vector-pathogen interactions and vaccine potential. The beagle is chosen for this study because it is the most commonly reported breed for similar studies in the literature and moreover, it is easy to work with this breed. Finally, this breed of dog is commercially available for use in experimental studies.

Project 4) Cattle are known to acquire Ehrlichia ruminantium infections naturally in endemic regions. Thus, they are highly susceptible to Heartwater disease and is ideally suited to define if the disease can be a risk for the US cattle industry.

3. Justify Animal Numbers

Justify numbers of animals to be used (attach timeline or flow chart and power analysis, if possible, to describe study groups). This section should include a description of animals used for colony maintenance (breeders and all offspring produced) as well as a description of experimental animal numbers. Total numbers should match the requested numbers in the species section.

- Animal Numbers Justification
- The Logical Determination of “N” in Animal Experimentation
- Non-Statistical Approach for Calculating the Optimum Number of Animals Needed in Research
- Statistics and the Issue of Animal Numbers in Research
- JUSTIFY ANIMAL NUMBERS EXAMPLE

Sample size calculation was performed to identify necessary sample size to distinguish between treatment groups accounting for repeated measures over time. Type 1 error at 5% and type 2 error rate set at 20% (80% power). Calculations were performed for differences in percent of T-cells producing interferon, PCR positives assessed by conventional and real time PCR assays, and to measure antibody levels. The largest sample size required was to detect differences requiring 6 dogs in each group to detect the expected differences in pathogenesis, pathogen persistence monitoring, and to differentiate between vaccinated animals and non-vaccinated controls over time. We also will include both sexes to account for variations resulting from sex as a variable. If an experiment is repeated multiple times, then the number of animals will be reduced to account for prior data as the way of justifying the reduced numbers; more details provided in the experimental design section.

6. Animal Husbandry

1. Facilities

In which animal facility will animals be housed?

	Facility
1	[redacted]
2	[redacted]

2. Housing Outside of Facility

Will animals be housed anywhere other than a designated animal housing facility for more than 12 hours (e.g., a laboratory)?

○ Yes ● No

3. Transportation Between Animal Housing/Use Facilities

Will animals be transported with a private vehicle between animal housing/use facilities?

○ Yes ● No

4. Non-Standard Husbandry

A. Does this protocol contain any Prolonged Physical Restraint?

See: ACUC Physical Restraint policy

○ Yes ● No

B. Does this protocol contain any Food/Fluid Regulation?

See: ACUC Food and Fluid Restriction policy

○ Yes
● No
○ Overnight only

C. Does this protocol contain Multiple Survival Surgical Procedures?

See: ACUC Multiple Survival Surgical Procedures policy

○ Yes ● No

D. Does this protocol contain any of the following Non Standard Husbandry?

- ☑ Single housing of social species
- ☐ Wire-bottom cages
- ☐ Special diet/water
- ☐ Extended time to weaning
- ☐ Extended time between cage changes
- ☐ Alternative light cycles
- ☐ Out of range temperatures
- ☐ Cage-size exceptions
- ☐ Other

i. Explain non-standard husbandry and list the length of time the animal will undergo non-standard husbandry.

When performing tick infestation studies, animals will need to be individually housed in their own pens, but at close proximity to each other. This will be important to minimize the damage to the tick cells placed on animals, while not adversely impacting the socialization of animals. Typically, tick cells will be on the animals up to about 7-10 days.

7. Description of Non-Surgical Procedures

1. Sample Collection

Will samples, such as blood or tissues, be collected from live animals? (Include sampling for genotyping.)

● Yes ○ No

A. Sample Type

Type of sample(s):

Mostly blood samples will be collected. In the event of animals requiring termination, such as in the RMSF and in heartwater disease infection studies (projects 3 and 4), tissue samples will be collected from several sources to define gross lesions, histopathological assessments and to look for the presence of pathogen by molecular or cell culture methods. These details were included in the project description.

B. Sample Volume

Volume of sample(s):

Sample volumes will be variable which vary from 1 ml to 20 ml. We provided additional details in the scientific project description section.

C. Sampling Frequency and Duration

Frequency of collection and for how long:

Maximum of 20 ml blood sampling occurs at times and when this happens it will only a once a week. Some times the blood volumes are 10 ml per draw and twice a week. Many times, 1 ml blood will be sampled. These volumes will be similar for dog and cattle studies we proposed. We do not anticipate drawing more than 40 ml of blood a week per animal.

D. Sampling Method

Method of collection:

Blood samples will be collected typically from jugular veins of dogs and cattle. We will also be sampling from cephalic and saphenous veins at times. The blood collections will not be carried out via intracardiac stick.

2. Induced or Spontaneous Neoplasia

Will induced or spontaneous neoplasia occur in live animals?

○ Yes ● No

3. Non-Surgical Procedures

	Procedure	Description of procedure	Building name	Room number or area
1	Tick transmission challenge in dogs	Infection challenge with tick transmission will be done as per our published protocol. Twenty-five adult infected tick pairs (25 males and 25 females) will be allowed to transmission feed on vaccinated dogs for 7 days. Engorged nymphs (obtained from a commercially available source) will be infected with E. chaffeensis, E. canis or A. phagocytophilum by needle inoculation and allowed to molt to the adult stage (Cheng et al. 2015 and Jaworski et al., 2016). To prepare for a tick transmission experiment, we will prepare a tick containment cell for each dog. In our system, we will use containment chambers constructed from the tops of Nalgene jars that are each fitted with a screen and polyvinyl gasket that will be directly glued (3M Scotch-Weld 4799 adhesive) to the shorn back of a dog. Dogs are manually held for the application of the tick containment cell. The shaved area will be approximately 4 inches in diameter and to either the right or left side of the dog over the midback area. The placement of containers will be done 24 hours prior to tick infestation. In addition, the dogs will be fitted with a collar to restrict grooming near the containment chamber. Tick infestations will be accomplished by placing 25 female and 25 male ticks on each dog. We will count ticks to be used for each dog carefully. The transfer of ticks to dogs will be performed by unscrewing the screened top of the container and placing the ticks on the dog. The top of the chamber will be re-secured immediately, and dogs will be returned to individual housing. The dog will be restricted from group play during the 7-day period that the tick containment cells are present. Dogs, tick containment chambers and tick attachments will be monitored daily until all ticks are removed from dogs. Extreme care will be taken, and all ticks will be counted (live or dead) when partially fed ticks are removed on day 7. The Nalgene top of the container will be removed from the polyvinyl gasket and the gasket will be removed by shaving. The dogs will be monitored for an additional four weeks.	[redacted]	to be decided
2	Tick feeding experiments with cattle	Acquisition feeding of ticks for both experiments 1 and 2: To determine if E. ruminantium can be acquired by A. maculatum, nymphs will be allowed to feed on all four groups of animals when we begin to see clinical signs or between 7 to 14 days post infection challenges. Ticks will be allowed to attach for feeding on steers (about 500 naïve nymphal ticks). Ticks will be allowed to secure complete blood meals and then allowed to molt to adult stages. Infection rates in the molted ticks will then be assessed by nested PCR analysis. During tick feeding, animals will be housed separately in pens as necessary and as per the CMG recommendation. Tick cells will be placed on steers. For these experiments, the backs of the animals will be shaved with veterinary clippers. A stockinette sleeve or hard capsule (cell) will be glued to the backs of steers. The firm attachment will be verified after about 24 h and prior to allowing ticks to feed. The cell will remain attached for several weeks. We will monitor twice daily for the retainment of the cell on the animals, as well as its firm attachment. To perform the tick infestation, ticks will be placed inside the cells and closed with the rubber bands or screw cap lid. Ticks will be collected following opening of the cell. We will try to account for all ticks on each animal by counting live and dead ticks.	[redacted]	to be decided

8. Substances Used in Animals

1. Substances Used in Animals

List the substances you will give the animals here (including vehicles given to controls, hazards, radiation, etc.):

	Substance	Amount/Dose/ Volume	Route	Frequency/ Duration	Hazard	Pharmaceutical Grade
1	Diphenhydramine	1mg per pound	oral	once before I.V. infections or vaccinations	No	Yes
2	Adjuvants	2.5% Montanide™ Gel	subcutaneous	twice	No	Yes
3	In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species	variable	I.V.	once	Yes	No
4	Naive and rickettsial bacteria infected ticks	25 pairs of adults of both sexes or 250 nymphs (for dogs) or 500 nymphs (cattle)	on the shaved surface of the skin	once	Yes	No
5	QS-21 saponin	1 mg	subcutaneous	twice	No	Yes
6	aluminium hydroxide	2%	subcutaneous	twice	No	Yes

2. Non-Pharmaceutical Grade Substances

For those substances that are marked “no” as pharmaceutical grade, list a justification in the space below. Also, include instructions for how they will be mixed to maintain sterility and adjust pH.

3. In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species used for infection studies will be obtained from our laboratory and are always grown in sterile culture conditions. Further, all procedures involving recovering the cultures will also be carried out using sterile experimental conditions.

4. Ticks are natural ectoparasites of animals. We will purchase them from a well-established tick rearing laboratory or maintained by us in the laboratory. It is not possible to obtain pharmacological grade ticks.

3. Substances Used in Animals Personal Protective Equipment (PPE)

PPE is needed to safely handle most materials in the laboratory. In general, a minimum of gloves and lab coat should be used. Other substances would require more PPE such as eye protection, respiratory protection, fume hood, etc. Please notify laboratory members if there are any special precautions that need to be taken when working with the above substances.

Describe the PPE required to handle these substances. You may group substances (e.g., “All substances” or “non-hazardous substances”) if all or some use the same PPE. Please list any substances needing alternative or additional PPE separately. You do not have to include additional PPE needed for work with hazards as that will be described in the Hazards section, however, you may include here as well if you wish.

	Substance	Gloves	Eye Protection	Lab Coat	Face Mask	Fume hood	Biosafety cabinet	Double-Gloves	Other	Other PPE
1	In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species	☑	☐	☑	☐	☐	☐	☑	☐	
2	Naive and rickettsial bacteria infected ticks	☑	☐	☑	☐	☐	☐	☑	☐	

Hazardous Agent

If you marked "yes" under Hazard, please complete the "Hazardous Materials" Section that follows.

9. Hazardous Materials

1. Will you use any Biological Hazards?

● Yes ○ No

A. Biological Hazard

List all biological hazards that will be used in live animal work.

	Agent or type of hazard	Donor species	Receiving species	Dose	Route/ Volume of Admin.	Frequency of Admin.	Other
1	In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species	N/A	Canine and bovine	2-5X10^8 organisms/ mL	I.V.	Once	
2	Naive and rickettsial bacteria infected ticks	N/A	Canine and bovine	1-2X10^5 organisms/ mL	N/A	Once	

B. IBC Protocol Number (if applicable for recombinant DNA or biological materials)

List your IBC Approval Number or attach your current IBC application. (Include attachments in the attached files section.)

IBC application is submitted and currently under review.

☐ Unsubmitted

☑ Submitted

☐ Approved

C. Biological Hazard - Anticipated Effect(s)

List any anticipated effect(s) of biological hazards on animal.

In project 1, E. chaffeensis random mutant organisms will be used to infect dogs. Naive nymphal stage ticks will be used to acquisition feed on dogs.

In project 2, modified live attenuated vaccine (MLAV) of E. chaffeensis and similarly, E. canis and A. phagocytophilum MLAV will be used for testing the vaccine efficacies. Infection challenges will be performed with in vitro cultured live organisms or using infected ticks. All three pathogens cause only mild disease as detailed in the project description section.

In project 3, rickettsia rickettsii cultured organisms will be used for the infection experiments before or after vaccinations. Non-vaccinated and the pathogen infected animals will develop a severe disease which can be fatal. A severe form of the disease requires close monitoring and observation and guidance of a veterinarian. We expect vaccinated animals to be healthy.

In project 4, cattle will be infected with Ehrlichia ruminantium. The pathogen may or may not cause severe disease, although we will anticipate the likelihood of developing severe clinical signs.

In all projects, we will work closely with an attending veterinarian to ensure that animals are cared humanely.

D. Biological Hazard - Housing/Procedure Sites

Where do you anticipate housing/working with animals receiving hazardous or potentially hazardous biological agents? Coordinate with the facility manager then list building and room numbers below.

	Agent	Receiving species	Building	Room or Area	Housing	Procedure
1	Tick transmission of Ehrlichia ruminantium	bovine	[redacted]	Housing and procedures	☑	☑
2	I.V. and tick transmission infections of Ehrlichia, Anaplasma and Rickettsia species	canine	[redacted]	Housing and procedures	☑	☑

E. Biological Hazard - Animal Identification

Explain how animals treated with a biological hazard will be identified (ex. cage card, ear tag, etc.)

- ☐ Cage Card
- ☐ Chip
- ☑ Door Sign
- ☐ Other

F. Hazardous Agents or By-Products /Presence

The biological hazard or by-products may be present in which of the following?

- ☐ None
- ☐ Feces/Urine/Bedding
- ☐ Saliva
- ☑ Blood
- ☐ Aerosols

- ☐ Animal bite/scratch
- ☑ Animal carcasses/tissues
- ☐ Surgical site wound or sore
- ☐ Other

G. Biological Hazard - Personal Protection Equipment (PPE) and Engineering Controls

PPE to be worn when handling biological hazards. LIDR ABSL-3 includes protective suit, shoe covers, double gloves, full-face PAPR.

	Biological Hazard	Gloves	Eye Protection	Lab Coat	Double-Gloves	Face Mask	Biosafety cabinet	LIDR ABSL-3	Other	Other PPE
1	Naive and rickettsial bacteria infected ticks	☑	☑	☑	☑	☐	☑	☐	☐	

H. Additional Information

List additional information, i.e., special precautions for pregnant women, immunocompromised individuals, special handling, or storage, etc.

2. Will you use any Chemical Hazards?

○ Yes ● No

3. Will you use any Radiation Hazards?

○ Yes ● No

10. Anesthetic Procedures, Pain Control, Other Clinical Drugs

1. Anesthetics, Preanesthetics & Tranquilizers

Will any anesthetics, preanesthetics, or tranquilizers be used?

○ Yes ● No

2. Pharmaceutical Analgesia

○ Yes ● No

3. Non-pharmacologic control of pain

○ Yes ● No

4. Paralytic Agents

○ Yes ● No

5. Antibiotics and Other Agents

(Include any emergency drugs, fluids, etc. here)

● Yes ○ No

6. Antibiotics and Other Agents

List other agents such as antibiotics and other emergency drugs

	Species	Agent	Dose/Volume	Route	Frequency of Admin.
1	Dog	Doxycyclin	10 mg/kg	oral	once per day for four weeks

11. Description of Surgical Procedures

1. Surgical Procedures

Will there be any surgical procedures?

○ Yes ● No

12. Potential Pain or Physical Stress

Potential Pain and/or Distress

Note: Animal Welfare Act regulations define a painful procedure as "any procedure that would reasonably be expected to cause more than slight or momentary pain ... in a human being to which that procedure was applied, that is, pain in excess of that caused by injections or other minor procedures." Procedures reasonably expected to cause pain in the absence of anesthetics or pain relieving drugs should be considered to have the potential to cause pain even with the use of such drugs.

1. Potential Side-Effects and Adverse Health Effects

Describe any potential side-effects or anticipated adverse health effects of all procedures listed in the preceding sections: animal husbandry, description of non-surgical procedures, anesthetic procedures, and surgical procedures.

In projects 1 and 2, clinical signs following infection challenges with Ehrlichia chaffeensis, Ehrlichia canis or Anaplasma phagocytophilum typically include only mild fever (rise in only up to 1.5 C above body temperature). Although lethargy and joint pain are possible, based on our past research experience, we do not anticipate seeing these signs with the infections.

Clinical signs of RMSF in dogs (project 3) may include fever, nausea, vomiting, muscle pain, lack of appetite, edema, and rashes. The disease can progress rapidly to a life-threatening illness within two weeks in naive animals.

Clinical signs of Heartwater disease in cattle resulting from Ehrlichia ruminantium (project 4) may result in significant morbidity. A sudden rise in high fever (107° F) coupled with the loss of appetite, depression and increased respiratory rate are likely. Neurological disorders may follow the respiratory signs which may include excessive chewing movements, incoordination, head tilting, rigid posture and staggered walking with a high-stepping gait. Animals may also exhibit convulsions or be unable to get up. These nervous signs may progress to mortality within one to two days. It is also possible that the animals may not exhibit any nervous signs before progressing to life threatening illness.

Adjuvants in project 3 might possibly induce a reaction. We will closely monitor the animals for such reactions and will follow the guidance of a clinical veterinarian.

2. Assurance of Limited Discomfort and Pain

Describe how it is assured that discomfort and pain are limited to that which is unavoidable for the conduct of this experimentation.

Projects 1 and 2: Ehrlichia and Anaplasma species infections in dogs animals will be observed twice daily with once daily monitoring the body temperatures. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer.

Project 3: After Rickettsia rickettsii infection with I.V. and tick transmission following vaccinations and in control groups, animals will be monitored twice daily with once daily monitoring the body temperatures. While we do not anticipate serious clinical signs for the vaccinated groups, all non-vaccinated infection controls are expected to develop a severe clinical disease. Onset of signs for I.V. may occur within three days while tick transmission may take about a week. The clinical signs will include high fever, edema, lethargy and lack of appetite. We will closely monitor the animals' health and promptly communicate with the attending veterinarian for appropriate action particularly if exhibiting depression, lethargy for more than 24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. Infection control group animals developing severe disease will be requiring euthanasia to alleviate the pain and suffering. We will be following the guidance of the veterinarian regarding when this decision needs to be made. In the event, the animals will be euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) using a commercial euthanasia solution.

Project 4: Ehrlichia ruminantium infections in cattle will be observed twice daily with once daily monitoring the body temperatures. Upon the onset of symptoms, daily collections of 2 ml blood will be initiated. An attending veterinarian will be consulted for appropriate action if the animals appear seriously ill, such as exhibiting depression, lethargy for more than24 hours, changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer, increased heart rate of respiration, or any neurological symptoms. While it is unclear if cattle develop a severe disease with E. ruminantium, in the event we do observe cattle infected with the pathogen develop severe disease, they will be requiring euthanasia to alleviate the pain and suffering. We will be following the guidance of the veterinarian regarding when this decision needs to be made. In the event, such cattle will be euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) by captive bolt method.

3. Pain and Distress Form

Is there a Pain and Distress form associated with this protocol?

See: Painful or Distressful Procedures

● Yes ○ No

Please attach the form in the attachments section of this protocol.

A. Which experimental groups, procedures, or animals require the Pain and Distress form?

Project 3 involving non-vaccinated dogs receiving infection by needle infection and tick transmitted challenge with Rickettsia rickettsii.

Project 4 involving the assessment of parthenogenesis in cattle following infection with Ehrlichia ruminantium.

Note: Files were attached with the previous submission.

13. Disposition of Animals

1. Animal Disposition

Check all that apply

- [x] Adoption (See MU adoption policy)
- [ ] Market
- [x] Euthanasia
- [x] Transfer to different project, PI, or another institution
- [ ] Returns to breeding colony, herd, source, owner, or wildlife site
- [ ] Other

2. Euthanasia

Euthanasia Statement

As noted in the Guide, “Euthanizing animals is psychologically difficult for some animal care, veterinary, and research personnel, particularly if they perform euthanasia repetitively or are emotionally attached to the animals being euthanized (Arluke 1990; NRC 2008; Rollin 1986; Wolfle 1985). When delegating euthanasia responsibilities, supervisors should be sensitive to this issue.”

A. Method of Euthanasia

Select the method of euthanasia

- [ ] Inhalant agent
- [x] Physical Method without Anesthesia
- [ ] Physical Method with Anesthesia
- [x] Noninhalent Pharmaceutical Agent

B. Euthanasia Descriptions

	Species	Agent/Method	Dose/Volume	Route
1	Dog	Euthanasia will be performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA).	Fatal-Plus®, of volume 0.22 ml/kg (86 mg/kg of pentobarbital) will be administered.	I.V. injection
2	Cattle	Captive bolt method	N/A	stunner fires a retractable bolt against the animal's head, primarily into the animal's brain

C. Additional Explanation of Euthanasia Procedures

Include any additional explanation of euthanasia procedures here.

Animals will be checked for the lack of heart beat and breathing to confirm the euthanasia procedure worked accordingly.

D. Scientific Justification for Use

- [x] AVMA Approved Method
- [ ] Not AVMA Approved Method

E. Secondary (Physical) Means of Assuring Euthanasia

- [ ] Bilateral pneumothorax
- [ ] Cervical dislocation
- [ ] Decapitation
- [ ] Exsanguination
- [x] Removal of vital organs

☐ Other

14. Project Information

1.

Associate	Role	Responsibilities	OHSP Training	Animal Care & Use	Survival Surgery	P&D Training
Ganta, Roman Reddy [redacted]	Principal Investigator Authorized to order animals Access to view cages		☑ Jan 27, 2023	☑ Feb 1, 2023	☑ Feb 2, 2023	⊖
[redacted]	Co-Investigator Authorized to order animals Access to view cages	Euthanasia P&D assessment	☑ Jan 11, 2023	☑ Jan 18, 2023	☑ Jan 19, 2023	☑ Jan 23, 2023
[redacted]	Co-Investigator Authorized to order animals Access to view cages	Surgery Euthanasia P&D assessment	☑ Jan 18, 2023	☑ Jan 19, 2023	☑ Jan 19, 2023	☑ Jan 23, 2023
[redacted]	Co-Investigator Authorized to order animals Access to view cages		☑ Jan 19, 2023	☑ Jan 20, 2023	☑ Jan 20, 2023	☑ Jan 23, 2023
[redacted]	Co-Investigator		☑ Jan 25, 2023	☑ Feb 2, 2023	☑ Feb 2, 2023	⊖
[redacted]	Co-Investigator Authorized to order animals Access to view cages		☑ Jan 18, 2023	☑ Jan 19, 2023	☑ Jan 26, 2023	☑ Jan 23, 2023
[redacted]	Key Personnel		☑ Feb 1, 2023	☑ Feb 1, 2023	☑ Feb 1, 2023	⊖
[redacted]	Key Personnel		☑ Jan 19, 2023	☑ Jan 20, 2023	⊖	⊖
[redacted]	Key Personnel		☑ Jul 2, 2023	☑ Jul 2, 2023	⊖	⊖
[redacted]	Key Personnel		☑ Feb 10, 2023	☑ Oct 21, 2020	⊖	⊖
[redacted]	Key Personnel		☑ Jan 18, 2023	☑ Jan 18, 2023	☑ Jan 20, 2023	☑ Jan 23, 2023
[redacted]	Key Personnel		☑ Jun 1, 2023	☑ May 31, 2023	⊖	⊖

2. Training and Qualifications

Provide a description of the training and qualifications for each individual listed above under Protocol Associates. Provide adequate detail to allow the ACUC to determine if the individual has adequate training and experience with the species and procedures to perform their role proficiently. If they do not have prior training or experience, how will this be obtained?

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
1	Ganta, Roman Reddy	dogs, sheep, and cattle	Handling, bleeding, vaccine and tick experiments, and measuring temperature.	10 years with dog work in all listed procedures Four months of working with sheep for handling and bleeding, I.V. infections Two months of working with cattle; support help with animal handling	Full-time employee
2	[redacted]	Cattle, sheep, rabbits, barnyard fowls, and wildlife animals	Animal husbandry handling, blood sampling, temperature measurements, surgical procedures, vaccine and tick studies, and euthanasia.	Served as a registered veterinary technician in the State of Kansas 2017– 2022 Animal husbandry (etc) 10+ years Veterinary practice (technician) work with , small, exotic, and wildlife animals 3 years Trapping, hunting, and wildlife management on rural farm 10+ years. Cattle in research – 2 years Sheep in research – 1 year Dogs in research – 2 years Mice in research – 1 year Surgical experience (veterinary practice) many species – 3 years Tick and vaccine studies with animals; dogs, sheep and cattle - about 6 months with each species Euthanasia for two years.	[redacted]
3	[redacted]	Cattle and swine	Cattle; Less than a year of experience, collecting blood, performing routine health checks Swine; Less than a year of experience, Collecting blood, taking temperature, weighing, performing routine health checks	Three months each for all the listed procedures	[redacted]
4	[redacted]	Cattle, sheep and mice	Cattle: temperature measurement, report clinical signs, help collecting blood samples Sheep: handling, bleeding, temperature measurements Mouse: handling, mice mating, dissection, Peritoneal injection, bleeding (terminal blood collection : cardiac puncture), collect of organs, euthanize using carbon dioxide chamber	Cattle 2 years Sheep; 6 weeks Mouse 4 years	[redacted]

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
5	[redacted]	cattle, sheep, goat, dogs, cats, donkeys, horses, and pigs	Animal handling, blood and tissue sample collection, animal health monitoring, surgeries, euthanasia and necropsies.	As a trained veterinarian (DVM equivalent) and also worked in clinical practice with 10 years of experience on all listed procedures	[redacted]
6	[redacted]	dogs, sheep, and cattle	Dog - handling, temperature and blood sampling Cattle - help with blood sample collections	Dog - 2 years handling, temperature and blood sampling Cattle - 1 year	Full-time employee
7	[redacted]	dogs, sheep, and cattle	Animal handling, blood sample collection, tick studies, and animal health monitoring	Dogs; 8 years of experience with all the above procedures. Cattle; 2 years for the listed procedures Sheep; 2 years also for the above listed procedures	Full-time employee
8	[redacted]	40 years with cattle, 25 years with dogs, 40 years with ticks on animals.	Collaborate with us on tick-animal studies and animal bleeding and infection experiments at times. Also he will assist with animal handling.	40 years	Full-time employee
9	[redacted]	3 years with mice, six months each with sheep and rats.	Assists with animal handling and bleeding.	She doesn't have prior experience with dogs, but has experience with mice and sheep. She will be trained by one of our group members having high level experience prior to her helping with the projects.	Full-time employee
10	[redacted]	six months each with sheep and mice.	animal handling and bleeding.	She doesn't have prior experience with dogs, but has experience with mice and sheep. She will be trained by one of our group members having high level experience prior to her helping with the projects.	Full-time employee
11	[redacted]	25 years of working as a veterinarian handling various domestic animals; dogs, cattle, sheep, goats	Bleeding, handling, treatment	Bleeding 25 years Handling 25 years treatment 25 years	Courtesy appointment/ Adjunct

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
12	[redacted]	None	Assist in holding animals and sample collections	None; she will be trained by one of our experienced team members.	[redacted]

Training Requirements

Note: The ACUC required Basic Training can be found at: https://research.missouri.edu/acqa/. This training must be updated every three years in order to receive protocol approval.

Note: It is the Principal Investigator's responsibility to ensure that all persons listed in Protocol Associates above participate in the MU Occupational Health and Safety Program. See Section 7:020 MU Business Policy and Procedures Manual for details. For enrollment procedures visit the OHSP website.

3. Funding Source

What is the funding source for this project? (Note: If funded internally or by a non-peer-reviewing agency, a peer review of scientific merit may be required.)

- [x] PHS (NIH, CDC, FDA, NSF, NASA)
- [ ] DoD
- [ ] VA
- [ ] AHA
- [x] USDA
- [ ] Foundation/Industry
- [ ] Internal
- [ ] Other

15. Refinements or Literature Search

Attach relevant files in the attached files section.

1. Painful Procedures

Any procedure that may potentially cause more than momentary or slight pain or distress requires a literature search for animal alternatives.

Are you performing any procedures that may potentially cause more than momentary or slight pain or distress?

● Yes ○ No

2. USDA Covered Species

Does this protocol utilize animals covered by the Animal Welfare Act or assigned to Category E? (AWA covered species include all warm blooded animals except birds, rats of the genus Rattus, and mice of the genus Mus, bred for use in research, horses not used for research purposes, and other farm animals.)

● Yes, includes USDA covered species or Category E ○ No

3. Includes USDA covered species or Category E

Search for Animal Alternatives

In the literature search and in the written narrative, replacement by non-animal systems, reduction in numbers of animals and refinement of experimental methods (the three R's) must be addressed.

Provide at least two sources of information: one of these sources must be a scientific literature database; documented expert consultation may be used as one source of information.

If you are in the School of Medicine and need assistance with this item, please contact Rachel Alexander, HSL Research Support Librarian, at AlexanderRL@health.missouri.edu. Others can contact the Zalk Veterinary Medical Library, at MU CVM VetMed Library for help.

See also:

https://www.nal.usda.gov/awic/sample-searches
https://library.missouri.edu
Literature Search Help

A. Source 1: Literature Database

Complete the information below:

	Date of Search	Name of Database	Years Covered by Search	Keywords and Search Strategy
1	December 120 2022	Pubmed	1950 to current	For project 1) Searched Ehrlichia chaffeensis AND mutagenesis AND pathogenesis with or without the word dog For project 2) The following words in several combinations were searched; vaccine OR vaccines OR attenuated live vaccines AND Anaplasma AND Ehrlichia AND dogs For project 3) vaccine OR vaccines OR attenuated live vaccine OR attenuated live vaccines AND dog OR dogs OR canine AND Rickettsia OR Rocky Mountain spotted fever OR Rickettsia rickettsii AND Rocky Mountain spotted fever vaccine For project 4) Searched the following combinations and other variations of the words; (((Salivary Glands) OR (Salivary Gland)) OR (saliva)) AND ((((((heartwater) OR (heartwater disease)) OR (ehrlichia ruminantium)) OR (cowdria ruminantium)) AND (((cattle) OR (ruminant)) OR (ruminants))) AND ((((((amblyomma) OR (amblyomma maculatum)) OR (Gulf coast tick)) OR (gulf coast ticks)) OR (tick, gulf coast)) OR (ticks, gulf coast)))

B. Source 2: Literature Database

For the second source you may use a literature database search or an expert consultation (see following question).

	Date of Search	Name of Database	Years Covered by Search	Keywords and Search Strategy
1	December 20, 2022	CAB Direct	1920 to present	For project 1) Searched Ehrlichia chaffeensis AND mutagenesis AND pathogenesis with or without the word dog For project 2) The following words in several combinations were searched; vaccine OR vaccines OR attenuated live vaccines AND Anaplasma AND Ehrlichia AND dogs For project 3) vaccine OR vaccines OR attenuated live vaccine OR attenuated live vaccines AND dog OR dogs OR canine AND Rickettsia OR Rocky Mountain spotted fever OR Rickettsia rickettsii AND Rocky Mountain spotted fever vaccine For project 4) Searched the following combinations and other variations of the words; (((Salivary Glands) OR (Salivary Gland)) OR (saliva)) AND ((((((heartwater) OR (heartwater disease)) OR (ehrlichia ruminantium)) OR (cowdria ruminantium)) AND (((cattle) OR (ruminant)) OR (ruminants))) AND ((((((amblyomma) OR (amblyomma maculatum)) OR (Gulf coast tick)) OR (gulf coast ticks)) OR (tick, gulf coast)) OR (ticks, gulf coast)))

C. Source 2: Expert Consultation (alternative)

For the second source you may use a literature database search or an expert consultation. Documented expert consultation may be used as one source of information.

No Sources...

D. Animal Alternatives Narrative

Based on the information from the sources above, provide a written narrative of alternatives to procedures that may potentially cause more than momentary or slight pain or distress. The narrative should be such that the ACUC can readily assess whether the search topics were appropriate and whether the search was sufficiently thorough.

If a possible alternative was identified or is known, but will not be employed, discuss why.

For project 1 PubMed search yielded 13 citations and 7 of them represent the work we previously published. The remaining 6, included a review, and are unrelated to the work proposed in our study. There is no evidence of duplication of our current work with any published research including our previous research. CAB Direct search with the similar word search yielded only three citations and two of which were our previous articles and a review. Again, we found no evidence for duplication.

For project 2, despite the use of several combinations of the listed words yielding 278 citation on the PubMed search, there was no evidence of any published work reporting any data on similar topics as we planned in the current study. Specifically, description of vaccine development, particularly using the live attenuated versions of Ehrlichia and Anaplasma pathogens impacting people or dogs are non-existing. CAB Direct for a similar search did not result in the detection of published research related to our proposed goals.

For project 3, Pubmed search resulted in 92 articles; 23 of which are related to vaccine studies in the past. Our recent publication on the topic is among the identified publications (Alhassan et al. 2019, Infect Immun. 2019 Jan 24;87(2):e00628-18. doi: 10.1128/IAI.00628-18). This article summarizes all the work prior to our study. Notably, the prior research focused mostly on inactivated vaccines did not translate in outcomes research for the RMSF vaccine development. The review article Richards [Expert Rev Vaccines. 2004 Oct;3(5):541-55. doi: 10.1586/14760584.3.5.541] is among the articles found. It

summarizes the importance of our study as it stated that the vaccine studies in the past century to prevention of rickettsial diseases did not yield any rickettsial vaccines manufactured and/or licenses. Also stated that "Early rickettsial vaccines were difficult, expensive and very hazardous to produce." Based on all these analyses, it is evident that the only significant publication related to vaccine studies is our recent publication. The current project, thus, extends our previous published work in developing vaccine that will likely be valuable for application for controlling the RMSF in dogs and possibly in people in the near future. Cab Direct found four results which included our above listed publication (Alhassan et al. 2019) and the remaining articles are unrelated the proposed project goals. Our prior publication indeed is the basis for expanding research on the current funded NIH grant for which this search was performed.

For project 4, a maximum of 9 citations were identified, but none of the publications were directly relevant to the project description we proposed. Thus, we will not be duplicating any prior studies.

16. Investigator Assurances

1. ABSL-2 Assurance

I will provide training to the husbandry/veterinary staff at least 48 hours prior to exposing animals to a biohazard regarding (but not limited to): the health hazards and symptoms of the biohazard(s) being used; husbandry related research specific SOP's (e.g. handling live exposed animals and contaminated cages); and animal/carcass disposition.

- ● Yes, I will meet the requirements of this statement.
- ○ No, I will not meet the requirements of this statement.
- ○ Not Applicable

2. Investigator Assurances

- ☑ 1. The information provided herein is accurate to the best of my knowledge.
- ☑ 2. Procedures involving vertebrate animals will be performed only by trained or experienced personnel, or under the direct supervision of trained or experienced persons.
- ☑ 3. Any change in the care and use of vertebrate animals involved in this protocol, will be promptly forwarded to the MU ACUC for review; such changes will not be implemented until the committee's approval is obtained.
- ☑ 4. The number of animals proposed is the minimum necessary to conduct valid experimentation.
- ☑ 5. I assure that I am not unnecessarily duplicating previous experiments.
- ☑ 6. I have considered alternative methods to using animals.
- ☑ 7. I understand that animal housing must be coordinated with the facility veterinarian and/or facility manager and that approval of this protocol does not guarantee space to house animals.

2023-10-10 10:39:51 -0500

Pain and Distress Form

PI: Ganta Protocol #: 41056 Species: Canine

Expected Clinical Signs (phenotype, disease, response to manipulations, etc.):

Rickettsia rickettsii can cause severe disease in non-vaccinated animals. Possible clinical signs include fever, nausea, vomiting, muscle pain, loss of appetite, edema, and skin rashes. The disease can progress rapidly to a life-threatening illness within two weeks in naïve animals.

Scoring Initiation (criteria or time when scoring will start):

Scoring will start the day following infection (either via injection or tick exposure) with *R. rickettsii.*

Scoring Frequency and Duration:

Frequency: If score < 0.5, score once daily
If score ≥ 0.5, score twice daily

Body weights will be performed at least once a week. On all other days, a body condition score (BCS) may be used to assess animal for evidence of weight loss.

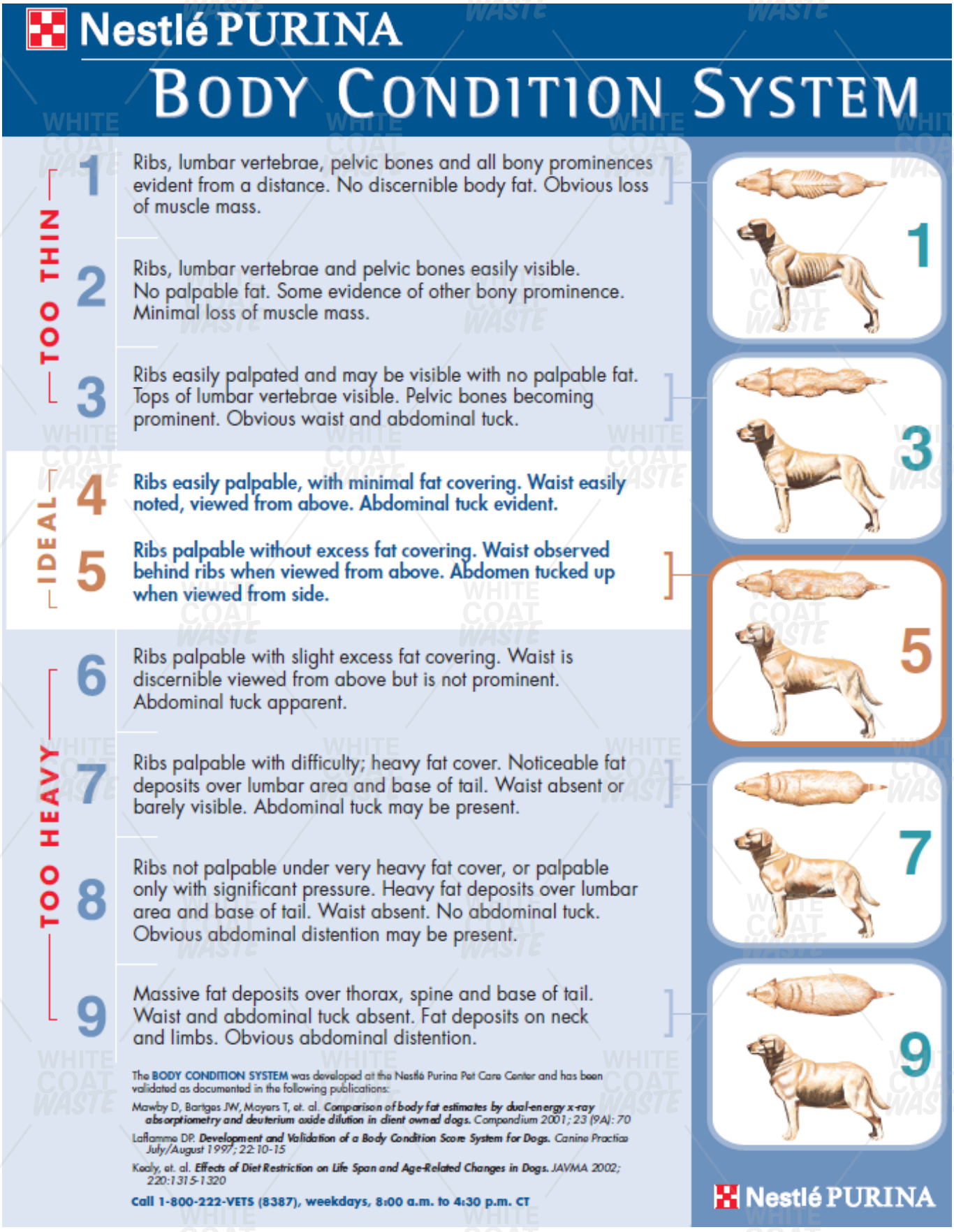
Duration: Scoring will be performed until euthanasia. After the first 14 days, if the body temperature is normal, body temperature frequency may be adjusted to once a week.

If total score ≥ 0.8, contact veterinarian
If total score ≥ 1.0, euthanize animal unless veterinarian permits a recheck*

*If an animal's total score is ≥1.0, the animal will be euthanized, or an OAR veterinarian must be notified to evaluate the animal. If the animal is determined to be in stable condition by the veterinarian, a recheck of the animal may be performed 8 hours later, or at an interval recommended by the veterinarian.

Observation	Score	Details
Attitude	0.0	BAR (Bright/Active/Responsive)
	0.2	Quiet but alert and rouses when approached or touched
	0.6	Lethargic, slower to rouse, may vocalize or be reluctant to stand
	1.0	Recumbent and minimally responsive
Weight Loss or Body Condition Score (BCS)	0.2	<5% weight loss OR BCS 4-9 / 9 (ideal body condition or overweight)
	0.6	10-20% weight loss OR BCS 3 / 9 (thin, bones can be felt with slight pressure and may be visible)
	1.0	>20% weight loss OR BCS ≤ 1-2 / 9 (very thin, bones can be felt easily and are visible)
Temperature	0.0	<103.5°F
	0.2	≥103.5 but <105°F
	0.4	≥105 but <106°F
	1.0	≥106°F
Appetite	0.0	Eating and drinking normally, appears hydrated (skin does not tent)
	0.2	Decreased food consumption, but appears hydrated (skin does not tent)
	0.8	Minimal food consumption and/or appears dehydrated (skin tents)

Figure 1. Body Condition Scoring in Dogs



Pain and Distress Form

PI: Ganta Protocol #: 41056 Species: Canine

Date	Animal #	Attitude Score	Weight Loss or BCS Score	Temperature Score	Appetite Score	Total Score	Action Taken	Scorer's Initials

Pain and Distress Form

PI: Ganta Protocol #: 41056 Species: Bovine

Expected Clinical Signs (phenotype, disease, response to manipulations, etc.):

Ehrlichia ruminantium may cause severe vascular endothelial damage in ruminants. Disease severity varies depending on ruminant species, breed, geographic origin, and strain of bacteria. Possible clinical signs include high fever, loss of appetite, depression, and increased respiratory rate. Neurologic signs such as excessive chewing movements, incoordination, head tilt, rigid posture, staggered walked or convulsions are also possible.

Scoring Initiation (criteria or time when scoring will start):

Scoring will start the day following infection (either via injection or tick exposure) with *E. ruminantium.*

Scoring Frequency and Duration:

Frequency: If score < 0.5, score once daily
If score ≥ 0.5, score twice daily

Duration: Scoring will be performed until euthanasia. After the first 14 days, if the body temperature is normal, body temperature frequency may be adjusted to once a week.

If total score ≥ 0.8 or neurologic signs present, contact veterinarian
If total score ≥ 1.0, euthanize animal unless veterinarian permits a recheck*

*If an animal's total score is ≥1.0, the animal will be euthanized, or an OAR veterinarian must be notified to evaluate the animal. If the animal is determined to be in stable condition by the veterinarian, a recheck of the animal may be performed 8 hours later, or at an interval recommended by the veterinarian.

Observation	Score	Details
Attitude	0.0	BAR (Bright/Active/Responsive)
	0.2	Quiet but alert and rouses/responds when approached or touched
	0.6	Lethargic, slower to rouse/respond, may vocalize or be reluctant to stand
	1.0	Recumbent and minimally responsive
Temperature	0.0	<103.5°F
	0.2	≥103.5 but <105°F
	0.4	≥105 but <106°F
	1.0	≥106°F
Respiration	0.0	Normal respiratory rate and depth
	0.4	Increased respiratory rate and/or effort, occasional coughing
	0.6	Labored breathing, and/or nasal discharge, frequent coughing
Appetite	0.0	Eating and drinking normally, appears hydrated (skin does not tent)
	0.2	Decreased food consumption, but appears hydrated (skin does not tent)
	0.8	Minimal food consumption and/or appears dehydrated (skin tents)

Pain and Distress Form

PI: Ganta Protocol #: 41056 Species: Bovine

Date	Animal #	Attitude Score	Temperature Score	Respiration Score	Appetite Score	Total Score	Action Taken	Scorer's Initials

University of Missouri

Animal Care Quality Assurance

[redacted]

January 17, 2025

Subject: IACUC APPROVAL

Dear Dr. Ganta,

Your MU animal use protocol **Protocol 41056 Amendment 8.1** entitled *"Tick-borne rickettsial diseases; pathogenesis and vaccine development"* was approved by the IACUC on January 17, 2025 and will expire on March 17, 2026.

Sincerely,

R. Scott Rector

Scott Rector, Ph.D.
Chair, Animal Care and Use Committee
Division of Research, Innovation & Impact

[redacted]

Pain and Distress Form

PI: Ganta Protocol #: 41056 Species: Canine

Expected Clinical Signs (phenotype, disease, response to manipulations, etc.):

Rickettsia rickettsii can cause severe disease in non-vaccinated animals. Possible clinical signs include fever, nausea, vomiting, muscle pain, loss of appetite, edema, and skin rashes. The disease can progress rapidly to a life-threatening illness within two weeks in naïve animals.

Scoring Initiation (criteria or time when scoring will start):

Scoring will start the day following infection (either via injection or tick exposure) with *R. rickettsii.*

Scoring Frequency and Duration:

Frequency: If score < 0.5, score once daily
If score ≥ 0.5, score twice daily

Body weights will be performed at least once a week. On all other days, a body condition score (BCS) may be used to assess animal for evidence of weight loss.

Duration: Scoring will be performed until euthanasia. After the first 14 days, if the body temperature is normal, body temperature frequency may be adjusted to once a week.

If total score ≥ 0.8, contact veterinarian
If total score ≥ 1.0, euthanize animal unless veterinarian permits a recheck*

*If an animal's total score is ≥1.0, the animal will be euthanized, or an OAR veterinarian must be notified to evaluate the animal. If the animal is determined to be in stable condition by the veterinarian, a recheck of the animal may be performed 8 hours later, or at an interval recommended by the veterinarian.

Observation	Score	Details
Attitude	0.0	BAR (Bright/Active/Responsive)
	0.2	Quiet but alert and rouses when approached or touched
	0.6	Lethargic, slower to rouse, may vocalize or be reluctant to stand
	1.0	Recumbent and minimally responsive
Weight Loss or Body Condition Score (BCS)	0.2	<5% weight loss OR BCS 4-9 / 9 (ideal body condition or overweight)
	0.6	10-20% weight loss OR BCS 3 / 9 (thin, bones can be felt with slight pressure and may be visible)
	1.0	>20% weight loss OR BCS ≤ 1-2 / 9 (very thin, bones can be felt easily and are visible)
Temperature	0.0	<103.5°F
	0.2	≥103.5 but <105°F
	0.4	≥105 but <106°F
	1.0	≥106°F
Appetite	0.0	Eating and drinking normally, appears hydrated (skin does not tent)
	0.2	Decreased food consumption, but appears hydrated (skin does not tent)
	0.8	Minimal food consumption and/or appears dehydrated (skin tents)

Figure 1. Body Condition Scoring in Dogs

Nestlé PURINA

BODY CONDITION SYSTEM

TOO THIN

1 Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance. No discernible body fat. Obvious loss of muscle mass.

2 Ribs, lumbar vertebrae and pelvic bones easily visible. No palpable fat. Some evidence of other bony prominence. Minimal loss of muscle mass.

3 Ribs easily palpated and may be visible with no palpable fat. Tops of lumbar vertebrae visible. Pelvic bones becoming prominent. Obvious waist and abdominal tuck.

IDEAL

4 **Ribs easily palpable, with minimal fat covering. Waist easily noted, viewed from above. Abdominal tuck evident.**

5 **Ribs palpable without excess fat covering. Waist observed behind ribs when viewed from above. Abdomen tucked up when viewed from side.**

TOO HEAVY

6 Ribs palpable with slight excess fat covering. Waist is discernible viewed from above but is not prominent. Abdominal tuck apparent.

7 Ribs palpable with difficulty; heavy fat cover. Noticeable fat deposits over lumbar area and base of tail. Waist absent or barely visible. Abdominal tuck may be present.

8 Ribs not palpable under very heavy fat cover, or palpable only with significant pressure. Heavy fat deposits over lumbar area and base of tail. Waist absent. No abdominal tuck. Obvious abdominal distention may be present.

9 Massive fat deposits over thorax, spine and base of tail. Waist and abdominal tuck absent. Fat deposits on neck and limbs. Obvious abdominal distention.

The **BODY CONDITION SYSTEM** was developed at the Nestlé Purina Pet Care Center and has been validated as documented in the following publications:

Mawby D, Bartges JW, Moyers T, et. al. ***Comparison of body fat estimates by dual-energy x-ray absorptiometry and deuterium oxide dilution in client owned dogs.*** *Compendium 2001; 23 (9A): 70*

Laflamme DP. ***Development and Validation of a Body Condition Score System for Dogs.*** *Canine Practice July/August 1997; 22:10-15*

Kealy, et. al. ***Effects of Diet Restriction on Life Span and Age-Related Changes in Dogs.*** *JAVMA 2002; 220:1315-1320*

Call 1-800-222-VETS (8387), weekdays, 8:00 a.m. to 4:30 p.m. CT

Nestlé PURINA

Pain and Distress Form

PI: Ganta Protocol #: 41056 Species: Canine

Date	Animal #	Attitude Score	Weight Loss or BCS Score	Temperature Score	Appetite Score	Total Score	Action Taken	Scorer's Initials

Protocol 41056 Amendment 8.1

Approval date	01/17/2025
Expiration date	03/17/2026

1. Basic Information

1. Elements ID

   For existing protocols, enter the ID assigned to this protocol in Topaz Elements.

2. eACUC Number (Automatically Assigned)

   41056

3. Principal Investigator

   Ganta, Roman Reddy

   Job title
   MCKEE ENDOWED PROFESSOR
   Department
   Veterinary Pathobiology
   Division
   Veterinary Medicine
   Business unit
   University of MO-Columbia

4. Protocol Title

   Tick-borne rickettsial diseases; pathogenesis and vaccine development

5. Triennial Re-write

   Is this protocol a triennial re-write of a protocol that was previously approved at the University of Missouri?

   ○ Yes ● No

2. Species Section

1. Please note, the total number of animals requested is the amount of animals you will need for a 3 year period. This number should include all experimental animals plus animals used for colony maintenance (breeders and offspring produced that are not used for experiments). These numbers should match the amounts in the Justify Animal Numbers section. If this is a triennial re-write these amounts should also include any animals on the previous protocol that will be transferred to the new protocol.

Species	Strain/ Stock/ Breed	Age/ Weight	Pain/Distress Category	Authorized	Ordered	Received	Adjustment	Available
Cattle	Holstein	18-24 months	Undefined (non-covered species only)	47				47
			USDA Category E	20				20
Total Cattles:				67	0	0	0	67
Dog	Beagle	6-10 months	USDA Category D	384	0	89		295
			USDA Category E	52	0	16		36
Total Dogs:				436	0	105	0	331

2. Phenotypic consequences

Describe any phenotypic consequences of the genetic changes to the animals and the outcome of these consequences (e.g. whether or not any change in animal welfare or husbandry is anticipated).

No Phenotypic consequences...

3. Wild Animals

Are WILD ANIMALS to be used or studied?

○ Yes ● No

4. Client-Owned Animals

Are CLIENT-OWNED animals to be used or studied?

○ Yes ● No

3. USDA Category E

1. Justification for Withholding Drugs

Provide scientific justification for withholding pain/distress-relieving drugs:

Non-vaccinated infection control animals in the RMSF study (project 3) will serve as controls to aiding to differentiate how effective the vaccine will be. If we start giving treatments to the control animals, we will not be able to make true comparisons of vaccine-associated protection, which is the primary goal of the study.

Primary objective of project 4 is is to define Heartwater disease parthenogenesis in the US cattle. We will need to record the infection severity as measured by clinical signs following infection with Ehrlichia ruminantium pathogen. Therefore, offering drugs to reduce the clinical signs will prevent us from assessing the disease severity.

2. Monitoring Pain and Stress

Explain how the level of pain or physical stress will be monitored (include the frequency of monitoring).

We will monitor all animals more closely; twice a day from the time we will fist observe clinical signs. In the event animals begin to show a severe disease symptoms, we will promptly contact the assigned veterinarian

for guidance. Accordingly, we may initiate supporting care such offering subcutaneous fluid therapy and/or other care as recommended, but not providing antibiotic treatments.

3. Point of Euthanasia

Define the point at which the animals will be euthanized.

The decision to euthanize animals will be as per the animal health status monitored and subject to recommendation of the veterinarian assigned to the project. Importantly, we will actively seek guidance regarding health status changes of animals and a decision will be made with a high priority given in providing humane treatment of animals.

4. Proposal Overview

1. Purpose

Purpose of the study:

To support the federally funded research grant proposals current in progress:

We currently have three active NIH funded R01 applications involving the use of animals; these studies involve the use of the canine host. We also have an active USDA cooperative agreement grant. This study will involve the use of cattle.

We currently have an NIH application which is reviewed and pending the funding decision for which we submit this revised application seeking an amendment; please see below the project number 5 for details.

1) NIH R01 grant # AI070908 (title: Vector and host contributions to the regulation of E. chaffeensis gene expression), we will need to perform in vivo screening of Ehrlichia chaffeensis mutants to identify genes essential for pathogens survival in vertebrate and tick hosts.

2) NIH R01 grant # AI152418 (title: Vaccines against Ehrlichia and Anaplasma species infections), canine host will be used to define the value of a modified live vaccine studies protecting against tick-borne rickettsial infections by Ehrlichia chaffeensis, Ehrlichia canis and Anaplasma phagocytophilum. Three primary goals (experiments) of this project are to; 1) evaluate the value of modified live attenuated vaccine (MLAV) to define the duration of immunity against wild type infection challenge through blood stream and tick transmission; 2) determine if immunity to MLAV protects against genetically distinct E. chaffeensis strains; and 3) to evaluate similar MLAVs from related Ehrlichia and Anaplasma species for their usefulness as a live attenuated vaccine protecting against infections. Goals of the first experiment are already accomplished during the year 1 and 2 funding, while experiments 2 and 3 are yet to be accomplished.

3) NIH R01 grant # AI152417 (title: Rocky Mountain Spotted fever vaccine development), we proposed to investigate the utility of whole cell inactivated vaccine to prevent Rocky Mountain spotted fever in dogs. This project major goals (experiments) involve the use of canine host; 1) evaluate inactivation methods for preparing WCA-S (Sheila Smith strain) and adjuvants in defining the vaccine protection; 2) evaluate the duration of immunity; 3) evaluate protection against tick-transmitted challenges; and 4) evaluate WCA protection against R. rickettsii heterologous strain infection challenges.

4) Ehrlichia ruminantium is an important foreign animal disease pathogen of ruminants as the infections with it in non-endemic regions can inflict major morbidity and mortalities. This sub-Saharan African pathogen is also well established in parts of the Caribbean islands. The goals of this proposal are to; 1) investigate heartwater disease pathogenesis in cattle resulting from an important tick-borne foreign animal disease pathogen in ruminants, Ehrlichia ruminantium; and 2) test if E. ruminantium can be transmitted by Amblyomma maculatum; the tick previously identified as a competent vector and having wide distribution in

southeastern parts of the USA.

5) NIH R01 grant application. Title: Genetics and axenic growth of tick-borne E. chaffeensis and A. phagocytophilum. In this project, we proposed to generate a random mutagenesis library spanning the majority of Ehrlichia chaffeensis genome (as part of Aim 1). The library of mutated bacteria will be evaluated by performing in vivo screening to identify genes essential for pathogens survival in vertebrate hosts. This experiment is essentially as described under project 1 and it will supersede the remaining portion of project 1, except that the study aims to expand the mutational library to broadly cover the entire E. chaffeensis genome. Further, we will not perform tick feeding studies on dogs. Thus, the in vivo screening experiments will be followed as we previously described under project 1, except that the tick attachment studies will be omitted.

6) Merck Animal Health and Rustici Rangeland and Cattle Research Endowment, University of California, Davis, CA. Title: Furthering research on bovine anaplasmosis vaccine as suitable for commercialization. In this project, we proposed to extend the modified live attenuated vaccine research 1) to assess its protection against heterogeneous mix of Anaplasma marginale strains and 2) determine the length of vaccine protection by mechanical and tick transmissions infection challenges.

2. Value

Please provide the information necessary to allow the ACUC to evaluate the objectives of the study against potential animal welfare concerns.

The studies in all four projects are independent and are critical for advancing our understanding of important tick-borne diseases impacting dogs, people and ruminants. The first project goals are to perform mutational analysis and in vivo screening to identify genes essential for the Ehrilchia chaffeensis pathogenesis in vertebrate and tick hosts. The second proposal aims to evaluate modified live vaccines against tick borne diseases in dogs and people resulting from E. chaffeensis, E. canis, and Anaplasma phagocytophilum. The 3rd project evaluates an inactivated whole cell antigen-based vaccine to confer protection against Rocky Mountain spotted fever (RMSF) which is a major fatal disease in dogs and people. The 4th project investigates pathogenesis of an important foreign animal tick-borne disease of ruminants. There are no non-animal alternatives for these tick-borne diseases. The objectives of the studies are the first to define pathogenesis and vaccine development in physiologically relevant animal models. All studies will be performed in accordance with the animal welfare regulations and the studies aim to develop the most effective methods to protect animals from several important tick-borne diseases which are more common in companion animals, agricultural animals and in people.

3. Lay Term Description of Experimental Design

To put something in layman's terms is to describe a complex or technical issue using words and terms that the average individual (someone without professional training in the subject area) can understand. This section should be written so that someone with a **10th grade science education can easily understand the project.**

The studies in all previously describe four projects and the pending NIH R01 application are independent and are critical for advancing our understanding of important tick-borne diseases impacting dogs, people and ruminants. The first project goals are to perform mutational analysis and in vivo screening to identify genes essential for the Ehrilchia chaffeensis pathogenesis in vertebrate and tick hosts; this project will be replaced with the new project 5. We will generate large pools of E. chaffeensis transposon mutants in support of this objective. The second proposal aims to evaluate modified live vaccines against tick borne diseases in dogs and people resulting from E. chaffeensis, Ehrlichia canis, and Anaplasma phagocytophilum. We recently developed a modified live attenuated vaccine which confers protection against infection challenge by direct blood-borne infection and against tick-transmission challenge. Specifically in the current project, we aim define the protection against heterologous strains of E. chaffeensis, and similarly test homologous modified live vaccines to protect dogs against E. canis and A. phagocytophilum infections. The 3rd project evaluates an inactivated whole cell antigen-based vaccine (WCAV) to confer protection against Rocky Mountain spotted

fever (RMSF) which is a major fatal disease in dogs and people. In our prior studies, we reported the best protect from WCAV and in the current study, we will assess various formulations of vaccine and length of protection using the best vaccine formulation; both against blood-borne infection, then test protection against tick transmission and finally against heterologous strains of the pathogen. The 4th project investigates pathogenesis of an important foreign animal tick-borne disease of ruminants; the heartwater disease caused by Ehrlichia ruminantium. This study investigates the risk of cattle from E. ruminantium by direct needle infection and from a tick native to the mainland USA, Amblyomma maculatum. The new NIH R01 application (project # 5) will be extension of the project #1 to perform in vivo screening of a random mutagenesis library. The most recent project focused on the bovine anaplasmosis modified live vaccine aims to extend vaccine protection against heterologous strains and to define the length of protection for up to one year against IV infection and from tick-transmission infection of a virulent A. marginale strain.

4. Scientific Description of Experimental Design

In language a scientific colleague can understand, provide a step-by-step, general description of the animal experiments you will perform including experimental groups and timing of procedures and manipulations. For complicated experimental designs, including a flow chart, diagram, or table in the Attachments section is recommended to help the ACUC understand what is proposed. DO NOT describe details of the procedures here as such details are requested later in the form.

Project 1) Active NIH grant # R01 AI070908: Vector and host contributions to the regulation of E. chaffeensis gene expression

Brief summary: Perform mutational analysis and in vivo screening to identify genes essential for the E. chaffeensis pathogenesis in vertebrate and tick hosts. We will generate large pools of E. chaffeensis transposon mutants in support of this objective. Our funding was approved to generate 200 mutant organisms. These mutants will then be screened to define the pathogenesis using the canine infection model; three experiments were proposed to accomplish this goal.

Background: The family Anaplasmataceae contains several obligate, intracellular, Gram-negative bacteria which include species of the genera Ehrlichia and Anaplasma and responsible for causing infections in dogs and people, as well as in several other vertebrate hosts. We recently performed mutational analysis and demonstrated that mutations in three different genes of E. chaffeensis caused attenuated growth of the organism in vivo (Cheng et al. 2013). These data formed the basis for our funded NIH-R01 grant application having the three specific aims. Aim 3 requires the use of animal studies, i.e., to perform mutational analysis and in vivo screening to identify additional genes essential for the E. chaffeensis pathogenesis in vertebrate and tick hosts. We have completed part the proposed experiments of this aim already at K-State as per an IACUC approval (Wang et al. 2020). This application will focus on the remaining proposed portion of the experiment. Dog is chosen as the infection model for the proposed experiments because it is an incidental host in acquiring E. chaffeensis similar to humans. Moreover, our several recent experimental studies demonstrated that this host serves as an excellent infection model, where the pathogen infection causing a very mild disease and the infection persists in (Nair et al. 2016). Our experimental infection studies demonstrated that dogs develop only mild fever (rise in only up to 1.5oC body temperature), while maintaining persistent infections with detectable hematological changes, host response and having milder histopathological changes.

Experimental plan:
Animal details. We will use about 6-month-old beagle breed dogs (representing both sexes equally) weighing approximately 8-10Kg for all of our studies. Animals will be purchased from a USDA approved vendor and acclimated for one week prior to introduction into the study. The study timeline and end points are described under each experiment.

Experiment involving animals: We proposed to screen 200 E. chaffeensis mutants in the canine host. As of now, we completed screening 60 mutants as 6 pools by infecting three dogs each with about 10 mutants in

each pool. A total of 18 were used under this objective as part of the current protocol at K-State. In this protocol, we will expect to screen 14 pools (maximum) of mutants to complete the project goals. Each pool of up to 10 mutants will be used and in three independent animals (n=3) per pool which totals 42 animals. The infection status will be assessed twice a week for two months. Nymphal ticks (typically about 250) will be allowed to acquisition feed on animals starting from day 5 post infection. Tick cells (containers that hold ticks) will be placed on dogs and covered with sheep soc (made of Nylon Spandex for easy flexibility) (Sheepman Supply co. or something similar) by following the procedures similar to those done on deer, except that there is no need for anesthetize the dogs. For these experiments, the backs of the animals will be shaved with veterinary clippers. A custom designed tick containment chamber (modified top of Nalgene jar containing screw cap lid) will be glued to polyvinyl membrane with a center circular opening. The chamber will then be glued to animals with industrial adhesive (commercially available). The chambers have round bottom smooth surface and once glued, the chambers remain attached for several weeks until polyvinyl membrane is lifted off the skin with the hair growth. To ensure that the chambers are tightly attached, tick infestations will be performed only after about 24 h following the attachment of the chambers. We will monitor for the retainment of the chambers on the animals, as well as their firm attachment. If dogs attempt to remove the chambers, we will place Elizabethan neck collars to restrict grooming. The chambers will be covered with sheep sox. To perform the tick infestation, lids of the chambers will be unscrewed, ticks will be placed inside, and the chambers will then be tightly closed with the lids and animals will be covered back with sheep sox. About 7 days following tick attachment, ticks will be collected by opening the chamber lids. We will evaluate ticks from each animal following the molting to adult stage to assess which mutants are acquired by ticks. Together, the assessments of blood (10 ml blood drawn twice a week from cephalic veins for the first two weeks and then on once a week) and tick sampling will help us determine which genomic regions of E. chaffeensis that are critical for the in vivo growth in an incidental host model with important implications in extending the observations in understanding pathogenesis in people (total dogs for this sub-experiment are 42).

Animal monitoring plan: After infection, animals will be observed twice daily with once daily monitoring the body temperatures. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.

Blood sampling and other procedures: In the experiments, animals will be kept for 60 days each to monitor the mutant E. chaffeensis circulation in blood. Blood sampling will be done twice a week from cephalic veins (10 ml each) for the first two weeks and then once a week thereafter. Total blood draws will be 11 times per animal. About 6- to 8-month-old dogs of the breed 'Beagle' will be used for these experiments. For convenience, we will either use all males or all females in each experimental group, while maintaining equal numbers of males and females throughout the study. The weight of each animal will be about 15 to 20 pounds. Diphenhydramine (Benadryl) (1mg per pound) will be orally administered to all animals about 30 minutes prior to inoculation with Ehrlichia. (The stock concentration to be used is 2.5 mg/ml; 6 to 8 ml per animal or 15-to-20-pound dogs.) Benadryl is administered to prevent any possible anaphylactic shock resulting from injection of organisms containing traces of serum or other animal products likely present in the culture media.

At the end of the study: At the completion of the study, dogs will be transferred to another study or will be adopted out after a four-week treatment with doxycycline. This infection is very common in dogs and pose milder disease and so it will not be a concern to either the dogs or to pet owners. The infection with E. chaffeensis is very common in dogs and poses milder disease (Bowman et al., 2009 and Beall et al. 2012). It will not be a concern to either the dogs or to pet owners. Thus, subjecting to adaptation or transferring to other research projects are fully justified. These animals will be transferred to other projects within the university as per the needs of a project(s) or may also be opened up for the adaption if such option is not

available.

Note: We will no longer be pursuing the studies planned as part of this project as the previously proposed mutational studies are sub-optimal and therefore the improved mutagenesis library generation and it use as described in the new NIH R01 grant proposal (project # 5) will be substituted. The proposed 42 dogs from this project, therefore, will no longer be used and so, we propose to reduce request from this project by 42 dogs. Therefore, the total number of requested dogs for the new project # 5, while are 80, we seek the approval request only for 38 more dogs (total # dogs for all the projects will be 384).

References:

Cheng C, Nair ADS, Indukuri VV, Gong S, Felsheim RF, Jaworski D, Munderloh UG and Ganta RR. Targeted and random mutagenesis of Ehrlichia chaffeensis for the identification of genes required for in vivo infection. PLoS Pathog. 2013 Feb;9(2):e1003171. doi: 10.1371/journal.ppat.1003171. Epub 2013 Feb 14.

Wang Y, Nair ADS, Alhassan A, Jaworski DC, Liu H, Trinkl K, Hove P, Ganta CK, Burkhardt N, Munderloh UG and Ganta RR. Multiple Ehrlichia chaffeensis genes critical for its persistent infection in a vertebrate host are identified by random mutagenesis coupled with in vivo infection assessment. Infect Immun. (2020) 88(10) DOI: 10.1128/IAI.00316-20.

Nair AD, Cheng C, Ganta CK, Sanderson MW, Alleman AR, Munderloh UG, Ganta RR. Comparative experimental infection study in dogs with Ehrlichia canis, E. chaffeensis, Anaplasma platys and A. phagocytophilum. PLoS One. 2016 Feb 3;11(2):e0148239. doi: 10.1371/journal.pone.0148239

Bowman, D., Little, S. E., Lorentzen, L., Shields, J., Sullivan, M. P., & Carlin, E. P. (2009). Prevalence and geographic distribution of Dirofilaria immitis, Borrelia burgdorferi, Ehrlichia canis, and Anaplasma phagocytophilum in dogs in the United States: results of a national clinic-based serologic survey. Vet Parasitol, 160(1-2), 138-148.

Beall MJ, Alleman AR, Breitschwerdt EB, Cohn LA, Couto CG, Dryden MW, Guptill LC, Iazbik C, Kania SA, Lathan P, Little SE, Roy A, Sayler KA, Stillman BA, Welles EG, Wolfson W, Yabsley MJ. Seroprevalence of Ehrlichia canis, Ehrlichia chaffeensis and Ehrlichia ewingii in dogs in North America. Parasit Vectors. 2012 Feb 8;5:29. doi: 10.1186/1756-3305-5-29. doi:10.1186/1756-3305-5-29

Project 2) Active NIH grant # R01 AI152418: Vaccines Against Ehrlichia and Anaplasma Species Infections

Brief summary: Tick-borne pathogens belong to the genera Ehrlichia and Anaplasma continue to emerge as a major public health concern during the last 3-4 decades. They include the emerging diseases; human monocytic ehrlichiosis, human ewingii ehrlichiosis, and human granulocytic anaplasmosis caused by Ehrlichia chaffeensis, Ehrlichia ewingii, and Anaplasma phagocytophilum. We recently reported the development of a modified live attenuated vaccine (MLAV) inactivating an important gene (ECH_0660) against E. chaffeensis that conferred protection against infection challenge from blood transfusion and from infected ticks (Nair et al. 2015 and McGill et al. 2016). Goals of this funded project are 1) to evaluate the duration of protection offered from E. chaffeensis MLAV against wild type infection challenge through blood stream and tick transmission; 2) to determine if immunity to the vaccine protects against genetically E. chaffeensis strains; and 3) to evaluate similar MLAV from related Ehrlichia and Anaplasma to protect against infections.

Background: Rickettsial diseases caused by pathogens of the Anaplasmataceae family, including members of the genera Ehrlichia and Anaplasma, are responsible for frequent infections in people over the past three decades and are a leading cause of tick-borne infections in humans throughout the USA and many parts of the world. These pathogens also infect diverse vertebrate hosts, although also are causing a milder disease in majority of host species. These pathogens have evolved strategies to evade host immunity and cause

persistent infections. Through our recently established mutagenesis experiments, we created E. chaffeensis mutants that contained insertions causing functional gene disruptions. An insertion mutation in the ECH_0660 gene resulted in the pathogen's rapid clearance from two vertebrate hosts (Cheng et al. 2013). Vaccination with this mutant induced a strong host response and offered complete protection against blood stream and tick transmission infection with wild-type E. chaffeensis one month after vaccination (Nair et al. 2015 and McGill et al. 2016). Previously, we performed molecular characterization of several E. chaffeensis isolates and reported that the isolates represent three distinct genetic groups (Cheng et al. 2003). We proposed the following three specific aims (all three involves the use of animals): 1) Evaluate the duration of immunity offered by the ECH_0660 gene mutant live attenuated vaccine (MLAV) against wild type infection challenge through blood stream and tick-transmission. 2) Evaluate the protection of the MLAV against genetically distinct E. chaffeensis strains. 3) Evaluate mutants in related Ehrlichia and Anaplasma species for their efficacy as live attenuated vaccines in conferring protection against the pathogens' infection into blood stream and by tick-transmission. As part of the completed research during the last two years, we completed the goals of aim 1, thus, we propose in executing experiments planned as part of aims 2 and 3 which we call as experiments 1 and 2.

Experimental plan:

Animal details. We will use about 6-month-old beagle breed dogs (representing both sexes equally) weighing approximately 8-10Kg for all of our studies. Animals will be purchased from a USDA approved vendor and acclimated for one week prior to introduction into the study. The study timeline and end points are described under each experiment.

Experiment 1: Evaluation of cross protection induced by MLAV against different E. chaffeensis strains
Experiment 1a) Comparison of Arkansas isolate-derived MLAV protection against St. Vincent and Jax infection challenges by I.V. and tick-transmitted infection

This experiment will have 8 groups (n=6); groups 1-4 will receive the Arkansas isolate derived MLAV intravenously, while Groups 5-8 will serve as infection controls. Groups 1 and 2 will receive I.V. infection challenge one month after vaccination with wild type St. Vincent and Jax culture infection challenges, respectively. As per our prior published data, infection challenge following one month of vaccination with attenuated mutant induce sufficient host immune response in offering complete protection against blood stream infection and tick transmission challenges with wild-type E. chaffeensis (1, 2). Groups 3 and 4 will be similar to Groups 1 and 2, except that the infection challenges will be performed by tick-transmission. Groups 5 and 6 (n=6) will serve as non-vaccinated controls but will be challenged via I.V. and Groups 7 and 8 (n=6) transmitted will be challenged tick- transmitted challenge with the St. Vincent or Jax isolates, respectively similar to groups 1-4 above.

Table 1. Experimental design to test Arkansas isolate-derived MLAV protection against St. Vincent and Jax infection challenges by I.V. and tick-transmitted infection

Group	Vaccine	# of animals*	Infection challenge.
1	Arkansas MLAV	1X I.V. 6 (3F+3M)	I.V.E. chaffeensis (St. Vincent)
2	Arkansas MLAV	1X I.V. 6 (3F+3M)	I.V.E. chaffeensis (Jax)
3	Arkansas MLAV	1X I.V. 6 (3F+3M)	tick transmission E. chaffeensis (St. Vincent)
4	Arkansas MLAV	1X I.V. 6 (3F+3M)	tick transmission E. chaffeensis (Jax)
5	Infection Control	6 (3F+3M)	I.V.E. chaffeensis (St. Vincent)
6	Infection Control	6 (3F+3M)	I.V.E. chaffeensis (Jax)
7	Infection Control	6 (3F+3M)	tick transmission E. chaffeensis (St. Vincent)
8	Infection Control	6 (3F+3M)	tick transmission E. chaffeensis (Jax)

*48 animals

Experiment 1b) Comparison of the St. Vincent isolate-derived MLAV protection against Arkansas and Jax

infection challenge by I.V. infection and by tick-transmission.
In this experiment, the St. Vincent isolate mutant MLAV will be used as the vaccine, and infection challenges will be performed with wild type I.V. infection and tick transmission with Arkansas and Jax isolates of E. chaffeensis. This experiment will have 4 vaccinated groups (n=6) and four non-vaccinated groups (n=6); groups 1-4 will receive the St. Vincent isolate derived MLAV intravenously, while Groups 5-8 will serve as infection controls. Infection challenges will be performed with wild type I.V. infection and tick transmission with Arkansas and Jax isolates. Groups 1 and 2 will receive I.V. infection challenge one month after vaccination with wild type Arkansas and Jax culture infection challenges, respectively. Groups 3 and 4 will be similar to Groups 1 and 2, except that the infection challenges will be performed by tick-transmission. Groups 5 and 6 (n=3) will serve as non-vaccinated controls and will receive I.V. infection challenge with the Arkansas or Jax isolate cultures, respectively. Groups 7 and 8 (n=3) will be challenged via tick-transmitted challenge with the Arkansas or Jax isolate infected ticks. Since we have sufficient number of control animals in the previous experiments, we reduced the number of control animals (n=3) in this study.

Table 2. Experimental design to test St. Vincent isolate-derived MLAV protection against Arkansas and Jax isolates infection challenges by I.V. and tick-transmitted infection
Group Vaccine # of animals* Infection challenge.
1. St. Vincent MLAV 1X I.V. 6 (3F+3M) I.V.E. chaffeensis (Arkansas)
2. St. Vincent MLAV 1X I.V. 6 (3F+3M) I.V.E. chaffeensis (Jax)
3. St. Vincent MLAV 1X I.V. 6 (3F+3M) tick transmission E. chaffeensis (Arkansas)
4. St. Vincent MLAV 1X I.V. 6 (3F+3M) tick transmission E. chaffeensis (Jax)
5. Infection Control 3 (F or M) I.V.E. chaffeensis (Arkansas)
6. Infection Control 3 (F or M) I.V.E. chaffeensis (Jax)
7. Infection Control 3 (F or M) tick transmission E. chaffeensis (Arkansas)
8. Infection Control 3 (F or M) tick transmission E. chaffeensis (Jax)

*36 animals

Experiment 1c) Comparison of the Jax isolate-derived MLAV protection against Arkansas and St. Vincent infection challenge by I.V. infection and by tick-transmission.
In this experiment, the Jax isolate mutant MLAV will be used as the vaccine, and infection challenges will be performed with wild type I.V. infection and tick transmission with Arkansas and St. Vincents isolates. This sub-aim will have four vaccinated groups (n=6) and all four groups will receive the MLAV and will also include four non-vaccinated control groups (n=3). Groups 1 and 2 will receive I.V. infection challenge one month after vaccination with wild type Arkansas and St. Vincents culture infection challenges, respectively. Groups 3 and 4 will be similar to Groups 1 and 2, except that the infection challenges will be performed by tick-transmission with the respective isolate infections. Groups 5 and 6 (n=3) will serve as non-vaccinated controls challenged via I.V. and Groups 7 and 8 (n=3) will be challenged via tick-transmitted challenge with Arkansas or St. Vincent isolates, respectively, similar to groups 3 and 4.

Table 3. Experimental design to test Jax isolate-derived MLAV protection against Arkansas and St. Vincent isolates infection challenges by I.V. and tick-transmitted infection

Group Vaccine # of animals Infection challenge .
1. Jax MLAV 1X I.V. 6 (3F 3M) I.V.E. chaffeensis (Arkansas)
2. Jax MLAV 1X I.V. 6 (3F 3M) I.V.E. chaffeensis (St. Vincent)
3. Jax MLAV 1X I.V. 6 (3F 3M) tick transmission E. chaffeensis (Arkansas)
4. Jax MLAV 1X I.V. 6 (3F 3M) tick transmission E. chaffeensis (St. Vincent)
5. Infection Control 3 (F or M) I.V.E. chaffeensis (Arkansas)
6. Infection Control 3 (F or M) I.V.E. chaffeensis (St. Vincent)
7. Infection Control 3 (F or M) tick transmission E. chaffeensis (Arkansas)
8. Infection Control 3 (F or M) tick transmission E. chaffeensis (St. Vincent)

*36 animals

Experiment 2) Evaluation of related Ehrlichia and Anaplasma species MLAV for their efficacy in conferring protection against wild type infection in the blood stream and by tick-transmission.
Experiment 2a): Evaluation of Ecaj_0381 disrupted MLAV's ability to confer protection against E. canis infection by I.V. into blood stream and by tick transmission.
This study will have two vaccination groups (n=6) and both groups will receive the same E. canis MLAV. Group 1 will receive I.V. infection challenge, while Group 2 will receive tick-transmission infection one month after vaccination. Groups 3 and 4 (n=3) will serve as non-vaccinated controls, which will receive infection challenges similar to Groups 1 and 2. For the control groups also we will use n=6.

Table 4. Experimental design to test E. canis MLAV protection against E. canis infection challenges by I.V. and tick-transmitted infection

Group Vaccine # of animals* Infection challenge.
1. E. canis MLAV 1X I.V. 6 (3F 3M) I.V.E. canis (wild type)
2. E. canis MLAV 1X I.V 6 (3F 3M) tick transmission E. canis (wild type)
3. Infection Control 6 (M or F) I.V.E. canis (wild type)
4. Infection Control 6 (M or F) tick transmission I.V.E. canis (wild type)

*24 animals

Experiment 2b): Evaluation of Aph_0634 disrupted MLAV's ability to confer protection against A. phagocytophilum infection challenge by I.V. infection into blood stream and by tick transmission.

In this study, A. phagocytophilum Aph_0634 mutant MLAV will be used as the vaccine similar to the previous experiment. Infection challenges will be performed with a human isolate of A. phagocytophilum (HGA2) using the wild type cultured organisms for I.V. infection and using infected ticks. As in the previous experiment, this study will include two vaccination groups (n=6) and two non-vaccinated control groups (n=6).

Table 5. Experimental design to test A. phagocytophilum MLAV protection against A. phagocytophilum infection challenges by I.V. and tick-transmitted infection

Group Vaccine # of animals* Infection challenge .
1. A. phagocytophilum MLAV 1X I.V. 6 (3F 3M) I.V.A. phagocytophilum (wild type)
2. A. phagocytophilum MLAV 1X I.V 6 (3F 3M) tick transmission A. phagocytophilum (wild type)
3. Infection Control 6 (M or F) I.V.A. phagocytophilum (wild type)
4. Infection Control 6 (F or M) tick transmission A. phagocytophilum (wild type)

*24 animals

Mutant Live Attenuated Vaccines (MLAVs): The MLAVs contain either modified E. chaffeensis, E. canis or A. phagocytophilum in vitro cultured mutant organisms washed with PBS and resuspended in PBS at a dose rate of 2X108 organisms/mL. Vaccines will be administered as I.V. (1 mL/animal).

Infection challenge dose: Infection challenges will be performed with 2X108 bacteria grown in appropriate cell culture by I.V. inoculation method; we chose this dose as we previously reported in an infection model utilizing this dose (Nair et al., 2016). E. canis organisms will be quantified in the culture; the culture will be centrifuged to concentrate and remove the culture media and resuspended into 1x PBS to a final concentration of 2X108 bacteria per 1 ml for use in inoculation experiments.

Intravenous injections: Each dog will receive 1 ml of the inocula into left or right cephalic vein using a 23 G butterfly needle. The vaccination site will be aseptically prepared by shaving hair (approximately 2cm2) and

cleaning with 70% ethanol. To prevent any possibility of developing anaphylactic reactions, Benadryl (diphenhydramine) will be administered 30 min prior to any intravenous vaccine or challenge inoculum administration.

Tick transmission challenge: Infection challenge with tick transmission will be done as per our published protocol. Twenty-five adult infected tick pairs (25 males and 25 females) will be allowed to transmission feed on vaccinated dogs for 7 days. Engorged nymphs (obtained from a commercially available source) will be infected with E. chaffeensis, E. canis or A. phagocytophilum by needle inoculation and allowed to molt to the adult stage (Cheng et al. 2015 and Jaworski et al., 2016). To prepare for a tick transmission experiment, we will prepare a tick containment cell for each dog. In our system, we will use containment chambers constructed from the tops of Nalgene jars that are each fitted with a screen and polyvinyl gasket that will be directly glued (3M Scotch-Weld 4799 adhesive) to the shorn back of a dog. Dogs are manually held for the application of the tick containment cell. The shaved area will be approximately 4 inches in diameter and to either the right or left side of the dog over the midback area. The placement of containers will be done 24 hours prior to tick infestation. In addition, the dogs will be fitted with a collar to restrict grooming near the containment chamber. Tick infestations will be accomplished by placing 25 female and 25 male ticks on each dog. We will count ticks to be used for each dog carefully. The transfer of ticks to dogs will be performed by unscrewing the screened top of the container and placing the ticks on the dog. The top of the chamber will be re-secured immediately, and dogs will be returned to individual housing. The dog will be restricted from group play during the 7-day period that the tick containment cells are present. Dogs, tick containment chambers and tick attachments will be monitored daily until all ticks are removed from dogs. Extreme care will be taken, and all ticks will be counted (live or dead) when partially fed ticks are removed on day 7. The Nalgene top of the container will be removed from the polyvinyl gasket and the gasket will be removed by shaving. The dogs will be monitored for an additional four weeks.

Animal monitoring plan: After infection with live vaccines and after infection challenges, animals will be observed twice daily with once daily monitoring the body temperatures. Body weights will be measured twice a week. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.

Blood sampling: All blood collections will be done from jugular, or anterior cephalic or lateral saphenous veins using 20 or 22 gauge needles.

Vaccination phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week during vaccination phase. In addition, 1 ml of whole blood in EDTA tube will also be obtained for performing CBC analysis (once a week) for one month following vaccination. One ml of blood in EDTA tube will also be collected and used for checking the infection status twice a week for the first month. In experiment 1, after the first month of vaccination, about 20 ml blood will be collected once in every two weeks until challenge.

Challenge phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week until end point. In addition, 2 ml blood in EDTA tubes will be collected twice a week for assessing the systemic bacterial load and 1ml blood will be collected for CBC analysis. If dogs exhibit high fever or other clinical symptoms, additional 1 ml blood may be collected a third time in a week to monitor the infection status.

At the end of the study: At the completion of the study, dogs will be transferred to another study or will be adopted out after a four-week treatment with doxycycline. The infections with E. chaffeensis, E. canis and A. phagocytophilum are very common in dogs and pose milder disease and will not be a concern to either the

dogs or to pet owners. Thus, subjecting to adaptation or transferring to other research projects are fully justified.

References:
Nair ADS, Cheng C, Jaworski DC, Ganta S, Sanderson MW, Ganta RR: Attenuated Mutants of Ehrlichia chaffeensis Induce Protection against Wild-Type Infection Challenge in the Reservoir Host and in an Incidental Host. Infection and immunity 2015, 83(7):2827-2835.

McGill JL, Nair ADS, Cheng C, Rusk RA, Jaworski DC, Ganta RR: Vaccination with an Attenuated Mutant of Ehrlichia chaffeensis Induces Pathogen-Specific CD4+ T Cell Immunity and Protection from Tick-Transmitted Wild-Type Challenge in the Canine Host. PLoS One 2016, 11(2):e0148229.

Cheng C, Nair ADS, Indukuri VV, Gong S, Felsheim RF, Jaworski D, Munderloh UG and Ganta RR. Targeted and random mutagenesis of Ehrlichia chaffeensis for the identification of genes required for in vivo infection. PLoS Pathog. 2013 Feb;9(2):e1003171. doi: 10.1371/journal.ppat.1003171. Epub 2013 Feb 14.

Cheng C, Paddock CD, Reddy Ganta R: Molecular heterogeneity of Ehrlichia chaffeensis isolates determined by sequence analysis of the 28-kilodalton outer membrane protein genes and other regions of the genome. Infection and immunity 2003, 71(1):187-195.

Project 3) Active NIH grant # R01 AI152417: Rocky Mountain Spotted fever vaccine development

Brief summary: Rocky Mountain spotted fever remains a life-threatening tick-borne disease of people and continues to be a public health concern in the USA and several North, Central and South American countries. During the last two decades, reported RMSF cases continue to rise in parts of North America. This NIH funded application investigates RMSF vaccine development using a relevant animal-tick-pathogen infection model (dog and tick). At the completion of the project, we expect to have a fully developed vaccine useful in devising strategies to control the disease.

Background: Tick-transmitted rickettsial diseases of the genera Anaplasma, Ehrlichia, and Rickettsia remain a growing public health concern in the USA and many parts of the world. The diseases include one of the oldest known rickettsial diseases, Rocky Mountain spotted fever (RMSF) caused by Rickettsia rickettsii. RMSF remains a serious disease of people and dogs for about a century and continues to be a public health concern in the USA and several North, Central and South American countries resulting from a tick bite (Alvarez-Hernandez et al., 2017; Piranda et al. 2008; Labruna et al., 2009; Piranda et al., 2011; Drexler et al., 2017; Hatcher et al. 2018; Londono et al. 2019;) [4, 7-19]. Clinical signs of RMSF include fever, headache, nausea, vomiting, muscle pain, lack of appetite, and rash. The disease can progress rapidly to a life-threatening illness in untreated patients, resulting in high mortality rates ranging from 30-80% [4, 20]. During the last two decades, reported RMSF cases continue rising in parts of North America (Drexler et al. 2017; Tinoco-Gracia et al. 2018). Since dogs develop disease similar to people, a vaccine to prevent the disease in this host will most likely be effective in controlling the disease spread from wildlife, ticks and also infections from dogs to people. We recently demonstrated that whole cell inactivated antigens of R. rickettsii offer complete protection against virulent infection challenge in the canine host (Alhassan et al.; 2019). Our prior published work offers the strongest justification for the proposed detailed investigation for which we received NIH grant funding. The following are the proposed objectives.

1) Evaluate inactivation methods for preparing WCA-S (Sheila Smith strain) and adjuvants in defining the vaccine protection.
2) Evaluate the duration of immunity
3) Evaluate protection against tick-transmitted challenges.
4) Evaluate WCA protection against R. rickettsii heterologous strain infection challenges.

Experimental plan:
Three different inactivation methods will be used to prepare WCA-S (whole cell inactivated antigen from Sheila Smith strain); heat, formalin and hydrogen peroxide.

Animal details: Purpose bred beagle dogs (4-6 months old of both sexes), weighing approximately 8-10 kg, obtained from a Class A USDA vendor, will be housed in indoor climate-controlled facilities with ad libitum food and water and adequate spacing to allow regular exercise activities. They will be acclimated for one week prior to introduction into the study. The study timeline and end points are described under each experiment.

Experiment 1: Evaluate inactivation methods for preparing WCA-S and adjuvants in defining the vaccine protection.

Vaccine assessments with WCA prepared by three different inactivation methods and using three different adjuvants: In our recent study, we used 70 µg of heat inactivated whole cell antigens of R. rickettsii Shelia Smith strain diluted in PBS with final concentration of 2.5% Montanide™ Gel.This experiment will be performed similarly; 9 vaccination groups will be included (n=6 for each group; 3 males and 3 females). One group will receive only adjuvant (n=6 and two animals each per adjuvant) and then will be subjected to infection challenge to serve as infection controls. (Total number animals for this experiment will be 60.)

We will not include uninfected controls as we have ample data generated previously using such controls. Vaccines prepared with three inactivation methods (heat, formaldehyde and H2O2) and with three different adjuvants (Montanide gel, QS-21 saponin and Aluminum hydroxide) will be used in this experiment. Similarly, adjuvant only preparations will be administered to control groups. The vaccination protocol will be similar to our recent publication with a priming vaccination on day 0, booster vaccination on day 21 and I.V. infection challenge with 105 R. rickettsii Shelia Smith strain organisms recovered from embryonated chicken eggs on day ~50 (Alhassan et al., 2019). Infection progression will be monitored for 30 days. All dogs in all groups will be monitored daily for health, clinical and behavioral changes, and twice weekly for hematological changes by complete blood count analysis. Body weights will be measured once a week. Body temperatures will be measured twice a week during the vaccination phase and daily following infection challenges. Temperature assessments will be done at similar times each day. Blood sampling will be performed as per the description in our recent publication for CBC analysis, to evaluate T- and B-cell responses, and to monitor bacterial burden of circulating R. rickettsii. At the end of the experiment, the animals will be euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) using a commercial euthanasia solution. A full necropsy will be performed, and tissue samples will be assessed for gross pathology and histopathology, as in (Alhassan et al., 2019). While our preference is to do all the groups at one time, we will be able to do this experiment in two phases if we are limited by the constraints of the facilities available for housing. (Note: depending on the resource availability and personnel management, we may opt to perform this experiment as two parts.)

Note: A minor modification will be submitted prior to experiment 1 to provide the exact details of which formulations are to be used once the results have been obtained.

Table 1.
Group Vaccine vaccination date* # of animals **. Infection Challenge***
1. (Heat & Montanide gel) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
2. (Heat & QR-21 saponin) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
3. (Heat & Aluminum hydroxide) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
4. (Formaldehyde & Montanide gel). Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
5. (Formaldehyde & QR-21 saponin). Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
6. (Formaldehyde & Aluminum hydroxide) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
7. (H2O2 & Montanide gel) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii

8. (H2O2 & QR-21 saponin) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
9. (H2O2 & Aluminum hydroxide) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii
10. Infection control (2 per adjuvant) Day 0 and 21 6 (3F+3M) ~day 50 I.V. 105 R. rickettsii

*All vaccinations will be performed subcutaneously.
**60 animals
**The infection challenge will be performed for all 10 groups with Shelia Smith strain of R. rickettsii.

Experiment 2: Assess the duration of immunity of WCA-S prepared using the optimum vaccine formulation.

In this experiment, we will investigate the duration of immunity induced by WCA-S. We will select the best vaccine formulation (inactivation method and adjuvant) as per the results identifying the most efficacious in Experiment 1. The criteria for selecting the best vaccine formulation will be based on the data assessments comparing the protection in clearing the clinical disease coupled with immune response determined by comparing the results of three different vaccine preparations and adjuvants. All data will be assessed and discussed by our research team to reach this conclusion. If all three vaccines will yield similar results, then we will add economic costs to determine our chose for the next set of experiments.

Note: A minor modification will be submitted after the completion of Experiment 1, and prior to any remaining experiments, to provide which vaccine formulation will be used for the experiments.

We selected four time points for assessing the protection following the booster vaccination: 2, 4, 8 and 12 months. This experiment will include four groups (n=6) and a control group (n=4) to serve as non- vaccinated infection challenge group for comparing the protection. The reason that n=4 will be sufficient to serve in the control group as by this point we will have sufficient knowledge regarding RMSF in the dog model, which will be based on our prior work as well as the results generated from our previous experiment. (Total animals for this experiment will be 28.) Vaccination protocol will be followed as in the previous experiment. Similarly, all assays to assess the bacterial clearance, host immune responses, hematological parameters and pathological assessments will be followed as per the previous experiment, except that the infection challenge times will be different for each group. Peripheral blood and sera will be collected from the animals from all groups immediately prior to each challenge, as well as on different days post R. rickettsii challenge to evaluate cellular and humoral memory responses throughout the course of the study

Table 2.
Group Vaccine. Vaccine* days. # of animals** Infection challenge*** .
1. Vaccine formulation Day 0 and 21 6 (3F+3M) 12 months after vaccination; I.V. 105 R. rickettsii
2. Vaccine formulation Day 0 and 21 6 (3F+3M). 8 months after vaccination; I.V. 105 R. rickettsii
3. Vaccine formulation Day 0 and 21 6 (3F+3M). 4 months after vaccination; I.V. 105 R. rickettsii
4. Vaccine formulation. Day 0 and 21 6 (3F+3M). 2 months after vaccination; I.V. 105 R. rickettsii
5. Infection Control (no vaccination) 4 (2F+ 2M) infection with groups 1-4; I.V.105 R. rickettsii

*All vaccinations will be performed subcutaneously.
**28 animals
***The infection challenge will be performed with the Shelia Smith strain of R. rickettsii.

Experiment 3: Evaluate protection against tick-transmitted challenges.

In this experiment, we will investigate the efficacy of the WCA-S vaccine against tick-transmitted challenge with R. rickettsii Sheila Smith strain. We will use the optimized vaccine formulation (with inactivation and adjuvant formulation) for this experiment. Three groups of dogs will be used in the tick- transmission challenge experiments. Two groups will be used for tick transmission challenge (n=6), while the third group will be used for I.V. infection challenge. We will reduce the number of dogs to 4 in the 3rd group, as we anticipate having sufficient data already in place regarding the efficacy of the WCA vaccine against I.V.

infection challenge. (Total number of animals for this experiment will be 12.) The 1st and 3rd group will receive WCA primary and booster vaccinations as described above. The 2nd group will serve as the non-vaccinated and tick-transmission infection control group. Infection challenge will be performed one month after the final WCA immunization, or as per the optimum time point established in our time course experiment described above. Dogs in groups 1 and 2 will receive tick transmitted infection challenge by allowing 25 pairs of R. rickettsii-infected adult D. variabilis ticks to feed on the dogs for a week. The third group will receive an I.V. infection challenge with 105 R. rickettsia organisms. All assays to assess the vaccine protection will be similar as in the previous experiments.

We will use engorged D. variabilis nymphal ticks (within 24 - 48 h post blood meal) obtained from a commercial vendor {we typically use BEI Resources (Manassas, VA) and the Tick Rearing Facility of Oklahoma State University (Stillwater, OK)} to inject with chicken egg embryo-derived R. rickettsii organisms suspended in PBS at a concentration of 100 bacteria per micro liter. Needle puncture inoculation (with 26-gauge needle) will be placed into the ventral side of the ticks. Ticks will then be allowed to molt to adult stage at room temperature by exposure to 14 h light and 10 h dark cycle in a 96% humidity chamber [118]; we followed this protocol as part of several earlier studies. About 10 randomly selected ticks will be assessed for the infection rates using individually isolated genomic DNAs as templates for the nested PCR targeting to AdR2 gene of R. rickettsii [22]. This method, however, may not yield infected ticks and is the reason we proposed experiments in this application seeking approval to generate infected ticks following acquisition feeding on R. ricketsii-infected dogs (described in the experimental section).

Table 3.

Group Vaccine* # of animals** Infection challenge with Sheila Smith strain***

1. WCA vaccine; 0 and 21 days 6 (3F+3M) after 1 month; tick transmission R. rickettsii
2. Infection controls. 6 (3F+3M). tick transmission R. rickettsii
3. WCA vaccine; 0 and 21 days. 4 (2F+2M) after 1 month; infection by I.V.105 R. rickettsii

*All vaccinations will be performed subcutaneously.
**16 animals
**The infection challenge will be performed with the Shelia Smith strain of R. rickettsii.

Experiment 4: Evaluate WCA protection against R. rickettsii heterologous strain infection challenges.

Experiment 4.1: Compare Sheila Smith strain derived WCA protection against Morgan strain infection challenge. In this experiment, WCA will be prepared using the Sheila Smith strain R. rickettsii and primary and booster vaccinations will be performed as per previous experiment. The infection challenge will then be performed using the heterologous, virulent R. rickettsii Morgan strain by I.V. infection and tick transmission. This experiment will include four groups (n=6); Groups 1 and 2 will be vaccinated and Groups 3 and 4 will serve as non-vaccinated controls. Groups 1 and 3 will be challenged via I.V. infection with 105 R. rickettsii Morgan strain organisms, while Groups 2 and 4 will be challenged via tick-transmission using R. rickettsii Morgan strain infected D. variabilis. (Total number of animals for this experiment will be 24.) Infected ticks will be generated as outlined previously. All parameters to assess the bacterial clearance, host immune responses, hematological responses and pathological assessments will be performed as described under aim 1.

Table 4.1.

Group Vaccine* # of animals** Infection Challenge

1. Sheila Smith WCA vaccine. 6 (3F+3M) I.V.105 R. rickettsii (Morgan)
2. Sheila Smith WCA vaccine. 6 (3F+3M) tick transmission R. rickettsii (Morgan)
3. Infection Control. 6 (3F+3M) I.V.105 R. rickettsii (Morgan)
4. Infection Control. 6 (3F+3M) tick transmission R. rickettsii (Morgan)

*All vaccinations will be performed subcutaneously.
**24 animals

Experiment 4.2: Compare Morgan strain-derived WCA protection against Sheila Smith strain infection challenge.
Approach: All proposed experiments in this sub-aim will be similar to the previous sub-aim, except that we will use the Morgan strain to prepare the WCA vaccine, and dogs will be challenged with the Sheila Smith strain of R. rickettsii. For groups 3 and 4, we will use only 4 animals each, as we expect to have sufficient data related to this kind of controls. (Total number of animals for this experiment will be 20.)

Table 4.2.
Group Vaccine* # of animals**. Infection Challenge
1. Morgan WCA vaccine 6 (3F+3M) I.V.105 R. rickettsii (Sheila Smith)
2. Morgan WCA vaccine 6 (3F+3M) tick transmission R. rickettsii (Sheila Smith)
3. Infection Control 4 (2F+2M) I.V.105 R. rickettsii (Sheila Smith)
4. Infection Control 4 (2F+2M) tick transmission R. rickettsii (Sheila Smith)

*All vaccinations will be performed subcutaneously.
**20 animals

Experiment 4.3: Compare Iowa strain derived WCA protection against Sheila Smith and Morgan strains' infection challenges.
Approach: The experimental design and assessments to monitor vaccine protection will also be similar to the previous two experiments. Here, we will use Iowa strain for preparing the WCA. The experiment will include 8 groups; four groups will receive the Iowa strain WCA vaccine, and four groups will serve as non-vaccinated controls. For the four non-vaccinated control groups, we will have four animals each. We believe that n=4 will be sufficient for non-vaccinated infection control groups as we will have ample data from similar controls from previous two sub aims. Vaccinated groups will have 6 dogs each. (Total number of animals for this experiment will be 40.) Two groups will receive the Morgan strain infection via I.V. or by tick-transmission; the remaining two groups will be challenged with the Sheila Smith strain via I.V. or tick-transmission.

Table 4.3
Group Vaccine* # of animals** Infection Challenge
1. Iowa WCA vaccine 6 (3F+3M) I.V.105 R. rickettsii (Morgan)
2. Iowa WCA vaccine 6 (3F+3M) tick transmission R. rickettsii (Morgan)
3. Iowa WCA vaccine 6 (3F+3M) I.V.105 R. rickettsii (Sheila Smith)
4. Iowa WCA vaccine 6 (3F 3M) tick transmission R. rickettsii (Sheila Smith)
5. Infection Control 4 (2F + 2M) I.V.105 R. rickettsii (Morgan)
6. Infection Control 4 (2F + 2M) tick transmission R. rickettsii (Morgan)
7. Infection Control 4 (2F + 2M) I.V.105 R. rickettsii (Sheila Smith)
8. Infection Control 4 (2F + 2M) tick transmission R. rickettsii (Sheila Smith)

*All vaccinations will be performed subcutaneously.
**40 animals

Subcutaneous injections: Dogs receiving WCA vaccines in all the above outlined experiments will be administered subcutaneously. Total of 70 micro grams of antigen will be mixed with an adjuvant in a final volume of 500 micro liters (0.5 ml) and the entire vaccine will be administered once and at one site at the back of an on animal after shaving the inoculation site.

Infection challenges: Each dog will receive 1 ml the inoculum into left or right cephalic vein using a 23 G butterfly needle. The infection site will be aseptically prepared by shaving hair (approximately 2cm x 2cm) and cleaning with 70% ethanol. To prevent any possibility of developing anaphylactic reactions, Benadryl (diphenhydramine) will be administered 30 min prior to any intravenous vaccine or challenge inoculum administration.

Tick transmission challenge: Infection challenge with tick transmission will be done as per our published protocol. Twenty-five adult tick pairs (25 males and 25 females) infected with Sheila Smith strain or Morgan strain (as per the experiments outlined above) will be allowed to transmission feed on vaccinated dogs for 7 days. To prepare for a tick transmission experiment, we will prepare a tick containment cell for each dog. In our system, we will use containment chambers constructed from the tops of Nalgene jars that are each fitted with a screen and polyvinyl gasket that will be directly glued (3M Scotch-Weld 4799 adhesive) to the shorn back of a dog. Dogs are manually restrained for the application of the tick containment cell. The shaved area will be approximately 4 inches in diameter and to either the right or left side of the dog over the mid back area. The placement of containers will be done 24 hours prior to tick infestation. In addition, the dogs will be fitted with a collar to restrict grooming near the containment chamber. Tick infestations will be accomplished by placing 25 female and 25 male ticks on each dog. We will count ticks to be used for each dog carefully. The transfer of ticks to dogs will be performed by unscrewing the screened top of the container and placing the ticks on the dog. The top of the chamber will be re- secured immediately, and dogs will be returned to individual housing. The dog will be restricted from group play during the 7-day period that the tick containment cells are present. Dogs, tick containment chambers and tick attachments will be monitored daily until all ticks are removed from dogs. Extreme care will be taken, and all ticks will be counted (live or dead) when partially fed ticks are removed on day 7. The Nalgene top of the container will be removed from the polyvinyl gasket and the gasket will be removed by shaving. The dogs will be monitored for an additional four weeks.

Animal monitoring plan: After Rickettsia rickettsii infection with I.V. and tick transmission following vaccinations and in control groups, all animals will be monitored twice daily with once daily monitoring the body temperatures. Body weights will also be measured twice a week. While we do not anticipate serious clinical signs for the vaccinated groups, all non-vaccinated infection controls are expected to develop a severe clinical disease. Onset of signs for I.V. may occur within three days while tick transmission may take about a week. The clinical signs will include high fever, edema, lethargy and lack of appetite. We will closely monitor the animals' health and promptly communicate with the attending veterinarian for appropriate action particularly if exhibiting depression, lethargy for more than 24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.
Blood sampling: All blood collections will be done from jugular, or anterior cephalic or lateral saphenous veins using 20-22 gauge needles.

Vaccination phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week during vaccination phase for the first 30 days and then every two weeks thereafter. In addition, 1 ml of whole blood in EDTA tube will also be obtained for performing CBC analysis once a week) for one month following vaccination. One ml of blood in EDTA tube will also be collected and used for checking the infection status twice a week for the first month. In experiment 1, after the first month of vaccination, about 20 ml blood will be collected once in every two weeks until challenge.

Challenge phase: About 20 ml of blood (10 ml in ACD tube and 10ml in EDTA tube) will be collected once a week until end point. In addition, 1 ml blood in EDTA tubes will be collected alternate days for 10 days for assessing the systemic bacterial load and 1 ml blood will be collected for CBC analysis. From day 11 to 21, blood sampled twice a week for CBC and bacterial analysis. If any dogs exhibit high fever or other clinical symptoms, additional 1 ml blood may be collected on the days of clinical signs to monitor the infection status.

Euthanasia and tissue sample collection: All dogs will be sacrificed following the assessment four-week assessment following the infection challenge. Before euthanasia, approximately 50 ml blood will be collected from vein puncture. Euthanasia will be performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA). Specifically, commercial euthanasia

solution, Fatal-Plus®, of volume 0.22 ml/kg (86 mg/kg of pentobarbital) will be administered I.V. after the terminal bleed. The following tissue samples will be collected postmortem; spleen, liver, lymph nodes, lung, brain and bone marrow and they will be used for final detailed assessment of infection and gross pathology status.

References:

Alvarez-Hernandez G, Roldan JFG, Milan NSH, Lash RR, Behravesh CB, Paddock CD: Rocky Mountain spotted fever in Mexico: past, present, and future. Lancet Infect Dis 2017, 17(6):e189-e196.

Piranda EM, Faccini JL, Pinter A, Saito TB, Pacheco RC, Hagiwara MK, Labruna MB: Experimental infection of dogs with a Brazilian strain of Rickettsia rickettsii: clinical and laboratory findings. Mem Inst Oswaldo Cruz 2008, 103(7):696-701.

Labruna MB, Kamakura O, Moraes-Filho J, Horta MC, Pacheco RC: Rocky Mountain Spotted Fever in Dogs, Brazil. Emerging Infectious Diseases 2009, 15(3):458-460.

Piranda EM, Faccini JL, Pinter A, Pacheco RC, Cancado PH, Labruna MB: Experimental infection of Rhipicephalus sanguineus ticks with the bacterium Rickettsia rickettsii, using experimentally infected dogs. Vector Borne Zoonotic Dis 2011, 11(1):29-36.

Drexler NA, Yaglom H, Casal M, Fierro M, Kriner P, Murphy B, Kjemtrup A, Paddock CD: Fatal Rocky Mountain Spotted Fever along the United States-Mexico Border, 2013-2016. Emerg Infect Dis 2017, 23(10):1621-1626.

Londono AF, Arango C, Acevedo-Gutierrez LY, Paternina LE, Montes C, Ruiz I, Labruna MB, Diaz FJ, Walker DH, Rodas JD: A Cluster of Cases of Rocky Mountain Spotted Fever in an Area Of Colombia Not Known to be Endemic for This Disease. Am J Trop Med Hyg 2019, 3(10):18-1007.

Tinoco-Gracia L, Lomeli MR, Hori-Oshima S, Stephenson N, Foley J: Molecular Confirmation of Rocky Mountain Spotted Fever Epidemic Agent in Mexicali, Mexico. Emerg Infect Dis 2018, 24(9):1723-1725.

Hatcher C, Karahalios B, Badam M: Septic Shock Caused by Rocky Mountain Spotted Fever in a Suburban Texas Patient with Pet Dog Exposure: A Case Report. Am J Case Rep 2018, 19:917- 919.

Alhassan A, Liu H, McGill J, Cerezo A, Jakkula LUMR, Nair ADS, Winkley E, Olson S, Marlow D, Sahni A et al: Rickettsia rickettsii Whole-Cell Antigens Offer Protection against Rocky Mountain Spotted Fever in the Canine Host. Infection and Immunity 2019, 87(2):e00628-00618.

Project 4) Active USDA cooperative agreement grant:

Brief summary: Ehrlichia ruminantium is the disease-causing agent for an important tick-transmitted foreign animal disease, Heartwater. The goals of this project are to test if the pathogen can be transmitted by an indigenous US vector tick; Amblyomma maculatum (Gulf Coast tick). Secondly, we propose to investigate if tick feeding, and salivary gland secretions can enhance virulence of E. ruminantium in cattle.

Background: Ehrlichia ruminantium, a tick-borne rickettsial bacterium, causes Heartwater disease in ruminants resulting in a severe vascular endothelial damage throughout sub-Saharan Africa and parts of the Caribbean (Marcelino et al. 2016). Subacute and subclinical forms of the disease inflict significant morbidity, while peracute and acute forms can cause high mortalities [2]. The disease severity varies greatly depending on ruminant species, the animal breeds and their geographic origins, and also for different E. ruminantium strains (Kasari et al.2010). Nearly two centuries ago, E. ruminantium and a major tick vector, Amblyomma variegatum (the tropical bont tick, also known as the Senegalese tick) from Sub-Saharan Africa were

introduced to certain Caribbean islands (Vachiéry et al. 2008). In our earlier studies, we reported the first molecular evidence to confirm the origins of E. ruminantium in the Caribbean to be from parts of northern Africa; Senegal and Sudan (Reddy et al. 1996). Despite the long presence of E. ruminantium (over two centuries) in three Caribbean islands in close proximity to each other (Guadeloupe, Antigua and Marie Galante) (Kelly et all, 2011), there is no obvious evidence of the pathogen spread and severe outbreaks (Barré et all 1995). However, the presence of the pathogen and a vector in parts of the Caribbean, coupled with the availability of potential indigenous vectors, such as Amblyomma maculatum (Gulf Coast tick), are identified as a major threat to the US ruminants. For the first time, we recently established a Heartwater research program on the mainland USA and performed the first infection study with seven different E. ruminantium strains (Nair et al. 2021). All sheep exhibited clinical signs characteristic of Heartwater disease, which included labored breathing, depression, coughing and nasal discharges. Gross pathology and histopathology observations in the animals were also consistent for Heartwater. However, the animals did not develop a severe form of disease. Specifically, we only observed subacute and subclinical disease with no progression to a fatal outcome (Nair et al. 2021). Much remains to be defined relative to the potential threat of the disease to the ruminants on the mainland USA.

The goals of this project are as follows: 1) Test if E. ruminantium can be transmitted by A. maculatum, the tick having wide distribution in southeastern parts of the USA. 2) Investigate if tick feeding and salivary gland secretions can enhance virulence of E. ruminantium in cattle.
Animals in use for all experiments: Steers of 6–12-month-old; 21 steers total will be obtained from a vendor.

Experiment 1: Determine if needle injected ticks will transmit E. ruminantium. We will generate infected ticks by following the needle infection method which we developed for other related ticks and rickettsial pathogens. We will use the infected adult A. maculatum (up to 25 pairs) for transmission experiments in one group of four steers to measure virulence. Up to 25 pairs of needle infected ticks will be allowed to feed to repletion upon each animal. Infection assessment will be followed for 60 days.

Experiment 2: Determine if saliva/salivary glands (saliva extracts) mixed with cultured E. ruminantium will enhance virulence.

2A: Uninfected A. maculatum adult ticks (n=100) will be partially fed 4-6 days and will be removed before repletion (which typically takes about 10-15 days) on an uninfected steer (n=1). Salivary extracts will be collected from the ticks. The steers will be either adopted out, transferred to another project, or sold back to a farm.

2B: We will mix saliva extracts with E. ruminantium cultured organisms for use in infection experiments in the following four groups of animals (n=4):

Group 1. Mix saliva extracts + cultured E. ruminantium (2 x 10^8 bacteria) and use it for subcutaneous inoculations (SQ).
Group 2. Cultured E. ruminantium (2 x 10^8 bacteria) using SQ inoculation alone.
Group 3. Mix saliva extracts + cultured E. ruminantium (2 x 10^8 bacteria) and use it for IV inoculation.
Group 4. Cultured E. ruminantium IV inoculation alone (2 x 10^8 bacteria). IV infections will be performed in 2 ml volume of the inocula into jugular veins or
subcutaneous injections as per animal grouping.

Sample Collection: For both experiments 1 and 2, blood samples will be collected from the jugular veins using a 20-gauge needle. Blood will be collected twice per week starting 2 days prior to the start of the experiment; 10 ml for use in monitoring CBC, culture and DNA analysis and for immunological studies. Two ml each of additional blood sampling will be done daily when animals exhibit fever and clinical signs. At the time of euthanasia, up to 100 mL will be collected from the jugular vein.

Acquisition feeding of ticks for both experiments 1 and 2: To determine if E. ruminantium can be acquired by

A. maculatum, nymphs will be allowed to feed on all four groups of animals when we begin to see clinical signs or between 7 to 14 days post infection challenges. Ticks will be allowed to attach for feeding on steers (about 500 naïve nymphal ticks). Ticks will be allowed to secure complete blood meals and then allowed to molt to adult stages. Infection rates in the molted ticks will then be assessed by nested PCR analysis. During tick feeding, animals will be housed separately in pens as necessary and as per the CMG recommendation. Tick cells will be placed on steers. For these experiments, the backs of the animals will be shaved with veterinary clippers. A stockinette sleeve or hard capsule (cell) will be glued to the backs of steers. The firm attachment will be verified after about 24 h and prior to allowing ticks to feed. The cell will remain attached for several weeks. We will monitor twice daily for the retainment of the cell on the animals, as well as its firm attachment. To perform the tick infestation, ticks will be placed inside the cells and closed with the rubber bands or screw cap lid. Ticks will be collected following opening of the cell. We will evaluate ticks from each animal following the molting to adult stage to assess E. ruminantium acquisition by ticks. We will try to account for all ticks on each animal by counting live and dead ticks.

Animal housing during tick feeding: Steers will be individually housed for the tick feeding experiments. Individual housing of the pens are necessary to prvent grooming of animls attempting to remove tick cells. Animals will be allowed to return to co-housing at the completion of tick feeding experiments, i.e., upon final tick removal which will take about 7-10 days.

Animal monitoring plan: After infection, animals will be observed twice daily with once daily monitoring the body temperatures. Upon the onset of symptoms, daily collections of 2 ml blood will be initiated. An attending veterinarian will be consulted for appropriate action if the animals appear seriously ill, such as exhibiting depression, lethargy for more than24 hours, changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer, increased heart rate of respiration, or any neurological symptoms.

After infection challenge: All animals in all groups will be monitored for clinical signs, hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis for the rickettsemia. All animals will be monitored for behavioral changes and any changes in their eating patterns. Body temperature will be measured daily for first two weeks and once a week thereafter until the end point of the study. Any abnormal changes noted in animals will be discussed with the CMG-assigned veterinarian for follow up action plans.

Euthanasia and tissue sample collection: All steers will be sacrificed at the end of the study by following the captive-bolt stunning method by a certified veterinarian (possibly by a VHC clinician; to be identified). Before euthanasia, approximately 100 ml blood will be collected. Euthanasia will be performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA). The following tissue samples will be collected postmortem; spleen, liver, lymph nodes, lung, brain and bone marrow and they will be used for final detailed assessment of infection and gross pathology status.

References:

Marcelino I, Holzmuller P, Stachurski F, Rodrigues V, and Vachiéry N. Ehrlichia ruminantium: the causal agent of heartwater. Chapter 13, pages 241-280. Book title: Rickettsiales: Biology, Epidemiology, Molecular Biology and Vaccine Development, Ed. Sunil Thomas (Ed.) (2016). Springer Publishers

Kasari TR, Miller RS, James AM, Freier JE. Recognition of the threat of Ehrlichia ruminantium infection in domestic and wild ruminants in the continental United States. J Am Vet Med Assoc. 2010 Sep 1;237(5):520-30. doi: 10.2460/javma.237.5.520.

Vachiéry N, Jeffery H, Pegram R, Aprelon R, Pinarello V, Kandassamy RLY, et al. Amblyomma variegatum ticks and heartwater on three Caribbean islands. Ann N Y Acad Sci. 2008;1149:191 -5. doi:10.1196/annals.1428.081.

Reddy GR, Sulsona CR, Harrison RH, Mahan SM BM, AF B, AF' S articles by 'Barbet, AF B. Sequence

heterogeneity of the major antigenic protein 1 genes from Cowdria ruminantium isolates from different geographical areas. Clin Diagnostic Lab Immunol. 1996;3:417 -22.

Kelly PJ, Lucas H, Yowell, Beati L, Dame J, Urdaz-Rodriguez J, Mahan S. Ehrlichia ruminantium in Amblyomma variegatum and domestic ruminants in the Caribbean. J Med Entomol. 2011 Mar;48(2):485-8. doi: 10.1603/me10172.

Barré N, Garris G, and Camus E. Propagation of the tick Amblyomma variegatum in the Caribbean. Rev Sci Tech. 1995 Sep;14(3):841-55. doi: 10.20506/rst.14.3.883.

Nair A., Hove P., Liu H., Wang Y, Cino-Ozuna A.G., Henningson J., Ganta C.K., and Ganta R.R. Experimental Infection of North American Sheep with Ehrlichia ruminantium. Pathogens 2021, 10, 451. https://www.mdpi.com/2076-0817/10/4/451

Project # 5: Genetics and axenic growth of tick-borne E. chaffeensis and A. phagocytophilum

Project summary pertaining to animal studies. Mutational studies to be performed with ease in all members of the rickettsiales, including for Anaplasmataceae and Rickettsiaceae family pathogens, continue to be a major limiting factor despite our previousely demonstrated progress (as part of the above listed project 1). With several years of continued research focused on developing mutagenesis methods, we made progress in laying the foundations for creating mutations in Ehrlichia and Anaplasma species. Despite our success in developing both random and targeted mutagenesis in E. chaffeensis aiding in defining pathogenesis, host and vector-specific differences in the bacterial functional gene requirements, and vaccine development (progress from the above listed projects #s 1 and 2), performing mutational experiments with ease require considerably more standardizations, particularly for use in broader research applications. In this proposed project, we planned the following specific aim (aim 1) of the three planned aims in which we proposed to use canine infection studies:

Specific aim 1) Optimize Himar1 mutagenesis supporting the development and evaluation of a mutational library spanning a greater portion of E. chaffeensis genome.

This aim has two sub-aims:
Sub-aim 1.1 research design involves improving the efficiency of random mutagenesis constructs promoting the generation of a large genome-wide coverage mutational ibrary spanning the E. chaffeensis genome; we anticipate generating a library having nearly 1,000 mutations. The library of random mutation clones of E. chaffeensis will then be screened as part of sub-aim 1.2 using the canine infection study.

Sub-aim 1.2) Screen the mutagenesis library to define functional significance of genes/proteins critical for the bacterial persistence in vertebrate and tick hosts by following the methods we described previously [1].

Prior research on the assessment of in vivo transposon mutant library screening using a rat infection study demonstrated that up to 80 mutants per pool can be screened for identifying genes essential for bacterial growth and survival [2-4]. Similarly, we have successfully used the in vivo screening strategy to assess E. chaffeensis mutants in the canine host, although we were extremely conservative in pooling only 10 mutants per pool [1]. The published literature pertaining to in vivo screening of a mutant library, including our work on E. chaffeensis, authenticate that our planned transposon mutagenesis library screening protocol will yield desirable outcomes. Further, our goal to use a physiologically relevant infection model is the closest to natural infection transmission occurring; E. chaffeensis also infects dogs and A. americanum tick is the same vector for causing infections in dogs and people.

To define the in vivo growth impact of E. chaffeensis mutations, pools of mutant organisms having 40

mutants per pool and total of 800 mutants (20 pools in all) will be evaluated in the canine host infection study and each pool of mutants will be tested in four animals. Thus, total number of dogs to be used in this study will be 80 (20 pools x 4 dogs per pool = 80). Infection progression with the pools and those persisting versus those eliminated in vivo will be determined for a 60-day infection assessment. Detailed experimental methods as described in our recent publication [1] will be followed in executing this sub-aim. Infection experiments in dogs will be performed in compliance with the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals (https://olaw.nih.gov/policies-laws/phs-policy.htm), the U.S. Department of Agriculture's (USDA) Animal Welfare Act & Regulations. At the end of each experiment, all animals will be treated with doxycycline in accordance with the ACUC recommendations, which are consistent with the recommendations of the Merk Veterinary manual (https://www.merckvetmanual.com/generalized-conditions/rickettsial-diseases/ehrlichiosis,-anaplasmosis,-and-related-infections-in-animals#Diagnosis_v3276820). About 6-8 month-old beagle dogs will be obtained from a USDA-certified commercial breeder. Dogs will be housed indoors at a climate-controlled animal facility at the CVM animal facility (as per the assigned location) and ad libitum feed and water will be provided. All dogs will be placed in pens with adequate space to allow regular exercise/activity. In addition, all dogs will be permitted to socialize in groups several times each day. The animals will be monitored for health and behavioral changes and twice weekly for body temperature and hematological changes during the study period. Veterinary care for the animals was overseen by a university veterinarian. E. chaffeensis mutants grown under in vitro culture conditions to about 80-90% infection will be harvested by centrifugation at 15,000 × g for 10 min at 4°C, supernatants will be discarded, and the cultures will be resuspended in 15 ml of 1× phosphate-buffered saline (PBS). The washing steps will be repeated twice, and the final cell pellet will be suspended to concentrate the cells to about 2 × 10^6 per ml, yielding an estimated concentration of ~2 × 10^8 Ehrlichia organisms per 1 ml. Equal volumes of the culture suspensions of randomly selected mutants will be mixed to create mutant pools having equal ratios of the mutants in each pool. One ml of each mutant pool per dog will be inoculated by IV injection and each mutant pool will be inoculated in four dogs. About 2 ml of blood will be recovered per dog into sterile EDTA tubes on day 0 (prior to infection) and twice a week starting from the day 3 post infection and until the end of 8 weeks. The blood samples will be used immediately or stored at 4°C until use (maximum of 1 day). The samples will be centrifuged at 3,000 rpm for 5 min, and buffy coats will be transferred to a 15 ml sterile Falcon centrifuge tubes containing 10 ml of erythrocyte (RBC) lysis buffer (155 mM NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA) and mixed several times until complete lysis of erythrocytes. The samples will then then centrifuged at 5,000 × g for 5 min. The buffy coat pellet from each sample will be mixed in 300 µl of 1× PBS. One-hundred-microliter volumes of the buffy coats recovered from blood samples will be used to recover total genomic DNA using the DNeasy blood and tissue kit (Qiagen, Germantown, MD). Purified DNA from each sample was dissolved in 200 µl of elution buffer. The DNAs will be used to assess E. chaffeensis infection status by performing nested PCR targeting the inserted fragment-specific DNA as we described previously reported (Wang et al. 2020). Samples testing positive for the insertion-specific DNA will be subsequently evaluated by nested PCRs targeting the transposon insertion fragment and the respective flanking genomic regions for the mutants using the insertion-specific primer sets. The assessments of blood (10 ml blood drawn twice a week from cephalic veins for the first two weeks and then on once a week) will help us determine which genomic regions of E. chaffeensis that are critical for the in vivo growth in an incidental host model with important implications in extending the observations in understanding pathogenesis in people.

Animal monitoring plan: After infection, animals will be observed twice daily with once daily monitoring the body temperatures. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than 24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. All animals will also be monitored for hematology and the presence of bacteria assessed by molecular methods, such as by PCR and culture recovery methods, as well as by blood smear analysis.

At the end of the study: At the completion of the study, dogs will be transferred to another study or will be adopted out after a four-week treatment with doxycycline. This infection is very common in dogs and pose

milder disease and so it will not be a concern to either the dogs or to pet owners. The infection with E. chaffeensis is very common in dogs and poses milder disease [5 and 6]. It will not be a concern to either the dogs or to pet owners. Thus, subjecting to adaptation or transferring to other research projects are fully justified. These animals will be transferred to other projects within the university as per the needs of a project(s) or may also be sent for the adaption if such option is not available (similar to our description under project # 1 and 2).

Refevernce:

1) Wang Y, Nair ADS, Alhassan A, Jaworski DC, Liu H, Trinkl K, et al. Multiple Ehrlichia chaffeensis genes critical for its persistent infection in a vertebrate host are identified by random mutagenesis coupled with in vivo infection assessment. Infection and immunity. 2020:IAI.00316-20; doi: 10.1128/iai.00316-20.

2) Himpsl SD, Lockatell CV, Hebel JR, Johnson DE, Mobley HLT. Identification of virulence determinants in uropathogenic Proteus mirabilis using signature-tagged mutagenesis. J Med Microbiol. 2008;57(Pt9):1068-78.

3) Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ. Identification of a virulenceassociated determinant, dihydrolipoamide dehydrogenase (lpd), in Mycoplasma gallisepticum through in vivo screening of transposon mutants. Infection and immunity. 2006;74(2):931-9; doi: 10.1128/iai.74.2.931-939.2006.

4) Jones AL, Knoll KM, Rubens CE. Identification of Streptococcus agalactiae virulence genes in the neonatal rat sepsis model using signature-tagged mutagenesis. Mol Microbiol. 2000;37(6):1444-55.

5) Bowman, D., Little, S. E., Lorentzen, L., Shields, J., Sullivan, M. P., & Carlin, E. P. (2009). Prevalence and geographic distribution of Dirofilaria immitis, Borrelia burgdorferi, Ehrlichia canis, and Anaplasma phagocytophilum in dogs in the United States: results of a national clinic-based serologic survey. Vet Parasitol, 160(1-2), 138-148.

6) Beall MJ, Alleman AR, Breitschwerdt EB, Cohn LA, Couto CG, Dryden MW, Guptill LC, Iazbik C, Kania SA, Lathan P, Little SE, Roy A, Sayler KA, Stillman BA, Welles EG, Wolfson W, Yabsley MJ. Seroprevalence of Ehrlichia canis, Ehrlichia chaffeensis and Ehrlichia ewingii in dogs in North America. Parasit Vectors. 2012 Feb 8;5:29. doi: 10.1186/1756-3305-5-29. doi:10.1186/1756-3305-5-29

Project #6: Furthering research on bovine anaplasmosis vaccine as suitable for commercialization.

Background: Bovine anaplasmosis resulting from Anaplasma marginale infections in RBCs is a major economically important disease throughout the world, including in the USA. The rickettsial bacterium is transmitted to cattle biologically by several species of hard ticks, as well as mechanical routes. Male ticks are primarily important in transmitting the pathogen, as they remain persistently infected and transmit during their repeated feeding cycles on cattle. The infection results in the heavy RBC breakdown leading to severe anemia and sudden death. Clinical signs of the disease include persistent high fever, icterus, weight loss, abortion in pregnant cows, decreased milk production in dairy cattle, and reduced meat production in beef cattle. The disease severity differs for different age groups; calves up to six months of age display no clinical signs and 6 to12 month animals develop milder disease, whereas high morbidities and mortalities can result in cattle over the age of one year. Animals recovered from the clinical disease remain persistently infected throughout their life and serve as reservoirs of infection in a herd. Furthermore, infected animals can acquire new infections with different strains of A. marginale despite recovering from the acute infection while having active persistent infection. The disease is identified as endemic in almost all parts of the world (https://idl-bnc-idrc.dspacedirect.org/handle/10625/58247) [1]. Bovine anaplasmosis is a major challenge due to lack of an effective vaccine. Several studies in the literature suggest that numerous strains of A. marginale exist in nature as assessed by MSP1a-based amplification and sequence analysis [2]. In recent assessment of cattle

blood samples collected from various California farms, we discovered increased correlation for the presence of A. marginale strains with increased infection prevalence (described in the preliminary data). The highest number of strains detected was about 15. Moreover, most common strains observed within a farm having high prevalence remained very similar. A vaccine must have the ability to protect against diverse strains. Considering the live A. centrale blood stabilate confers protection against A. marginale infections by both mechanical and tick-transmission challenge [1], we anticipate that our newly established MLAV offers strong protection against divergent A. marginale strains transmitted by ticks and by mechanical transmission. We reported recently that the functional disruption in the gene encoding the membrane-bound phage head-to-tail connector protein (phtcp) of A. marginale cause reduction of the systemic bacterial loads in a host, while inducing sufficient immune response providing protection against wildtype infection challenges [3,4]. Cattle infected with the A. marginale mutant as a live vaccine prevented the disease compared to wildtype pathogen infections resulting from IV infection and tick-transmission [3,4]. These exciting data authenticates that the continued development of an effective live vaccine is well-justified which allow defining the vaccine protection against heterologous strains and for the extended period of up to one year.

Experiment 1) Define the MLAV protection against heterogeneous strains of A. marginale by mechanical and tick transmitted challenges.

All experiments with cattle will be performed in accordance with the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals (https://olaw.nih.gov/policies-laws/phs-policy.htm), the USDA Animal Welfare Act & Regulations. Holstein steers, approximately 18 months old, will be obtained from an area (such as North Dakota) where bovine anaplasmosis is reported to be less prevalent. To confirm no prior exposure, whole blood from each animal will be screened by an MSP5-based cELISA (Anaplasma Antibody Test Kit, cELISA v2;VMRD, Pullman, WA, USA) and by A. marginale 16S rDNA qPCR, respectively [3]. The steers will be housed at a vector-free animal facility at University of Missouri with food and water provided ad libitum. Steers will be allowed to socialize within their respective groups' animals. Animals will be individually housed when tick studies are to be performed. Adequate space will be allotted allowing regular exercise/ activity for the steers.

Firstly, we prepare blood stabilates for the study (two steers will be used): Blood stabilate generation requires infecting a naïve steer with a culture stock/blood stabilate. For generating blood stabilates for two isolates, two steers will be inoculated and infection will be monitored for up to 60 days. Based on our prior studies and by other researchers, we anticipate having animals testing positive for infection starting one week post infection with peak bacteremia ~day 30 which correlates well with a drop in packed cell volume and occasional spike in fevers. Blood will be collected on alternate days when the bacteremia levels were about 11%, for use in preparing stabilates for infection experiments described below.

Two groups each of nonvaccinated and vaccinated animals (n=5 each) will be used in this objective (total steers will be 20). This sample size is selected based on an a priori power analysis using estimates of effects of MLAV on bacterial load which we observed in our recent vaccine study [4] where bacterial numbers were calculated using 16S rDNA qPCR. Inoculation with the vaccine for two groups will be performed using the in vitro cultured MLAV organisms (~3 x 108 ISE6 culture-derived organisms resuspended in 2 ml of 1X PBS). Wildtype A. marginale blood stabilates prepared from two heterologous strains (described in the previous paragraph) will be used as the infection inocula for I.V. infection group [2]. Animals from all four groups will be monitored for 80 days to assess clinical disease, changes in Complete Blood Count (CBC), including PCV, systemic infection status changes to blood cell morphology, antibody, T-cell, and cytokine responses. (Total number of steers for this experiment will be 22).

Experiment 2) Define the length of protection of the MLAV to prevent the clinical disease against A. marginale infection resulting from mechanical and tick transmissions.

Ticks are typically more active during summer months; thus, the pathogen infections and the disease prevalence is often seasonal with a surge in infections during this time. Considering these points, it is

important for a vaccine to be effective for a minimum of 6 months and a maximum of one year. This experiment aims to define the immune protection offered by our newly developed MLAV for up to one year. This experiment will be executed for defining the length of immune protection for 6 months and one year against virulent A. marginale homologous strain challenge by I.V. infection and by tick transmission. All parameters of immune protection will be assessed essentially as described in the previous objective. We will have 6 groups of animals in this study. Four vaccinated groups (n=4 per group) and two non-vaccinated groups (n=4 per group) (total number of steers for the 6 groups = 24). We reasoned that by this time, we will have sufficient data generated from vaccine assessment studies with MLAV (from our previous research and the planned research under experiment 1 and so reducing the number of animals from five to four will be sufficient for the vaccine groups. We will initially vaccinate two groups with the MLAV, and two additional vaccination groups will be introduced after 6 months to represent 6-month vaccine groups. After 12 months, we will bring additional animals for use in the nonvaccinated infection control groups. One group each from the 6-month and 12-month vaccinated animals will be I.V. infection challenged with the virulent blood stabilate A. marginale. The same strain-infected adult male ticks will be used to transmission feed for a week on the remaining two 6-month and 12-month vaccinated groups. Infected ticks will be generated by allowing ticks to feed on unvaccinated and infection control group steers essentially as described previously in [3]. To allow generating the infected for the tick transmission challenge, experiments will be performed sequentially. The clinical disease, hematological changes, host immune responses, and the infection progression will be assessed for 80 days following infection challenges in all 6 groups of animals similar to our prior studies, as outlined under objective 1. The data will be assessed to determine if the MLAV offers protection for 6 and/or for 12 months in steers. (Steers to be used for the project are: 22 for objective and 24 for objective which totals to 46).

Animal monitoring, CBC, and assessment of systemic A. marginale: All cattle in the study will be monitored daily for health and behavioral changes and twice weekly for body temperature which may be amended to daily monitoring if animals exhibit severe clinical signs. Veterinary care for the animals will be overseen by the University animal facility appointed veterinarian. About 10 ml blood will be collected in EDTA tubes once a week from all animals for plasma and DNA analysis. Additionally, ~1 ml blood samples will be collected daily throughout the acute phase of infection. Similarly, ~1 ml of blood will be collected twice a week for use in CBC analysis which will be performed on a IDEXX ProCyte Dx Haematology analyzer. Blood sampled from all animals will also be assessed for A. marginale numbers by 16S rDNA qPCR assay. All blood samples will be processed immediately or stored at 4°C for a maximum of 24 h prior to performing the described analyses. DNeasy Blood and Tissue DNA isolation kit (Qiagen, Germantown, MD) will be used to extract total genomic DNA from a 100 µl aliquot of the whole blood samples. Extracted genomic DNA from each sample will be assessed for A. marginale infection status by qPCR assay targeting the 16S rDNA. Likewise, the A. marginale-specific IgG production will be assessed by ELISA using in vitro culture-derived bacterial antigen coated plates. Cytokine and T-cell responses will be assessed in Peripheral Blood Mononuclear Cells (PBMC) stimulated assays.

References:

1. Kocan KM, de la Fuente J, Guglielmone AA, Melendez RD. Antigens and alternatives for control of Anaplasma marginale infection in cattle. Clin Microbiol Rev. 2003;16: 698–712. doi:10.1128/CMR.16.4.698
2. Lew AE, Bock RE, Minchin CM, Masaka S. A msp1α polymerase chain reaction assay for specific detection and differentiation of Anaplasma marginale isolates. Vet Microbiol. 2002;86: 325–335. doi:10.1016/S0378-1135(02)00017-2
3. Hove P, Madesh S, Nair A, Jaworski D, Liu H, Ferm J, et al. Targeted mutagenesis in Anaplasma marginale to define virulence and vaccine development against bovine anaplasmosis. PLOS Pathog. 2022;18: e1010540. doi:10.1371/JOURNAL.PPAT.1010540
4. Ferm J, Jaworski DC, Stoll I, Kleinhenz MD, Kocan KM, Madesh S, Ferm D, Liu H, Fitzwater S, Schlieper A, and Ganta RR. Genetically modified live vaccine offers protective immunity against wild-type Anaplasma marginale tick-transmission challenge. Vaccine, 2024 Oct 24;42(24):126069. doi: 10.1016/j.vaccine.2024.06.036.

5. Justify

1. Justify Use of Animals in your Research

   Justify the use of animals for your experimental goals. **DO NOT** describe details of the experimental design or justify animal numbers here.

   There are no non-animal alternatives for all four proposed projects. Investigations focused on pathogenesis and vaccine development studies require the use of animals, particularly those naturally acquire infections are the best to define and develop effective methods of control.

2. Justify Animal Species

   Justify the choice of species for your study.

   Projects 1, 2, and 5) dog is the perfect animal model for such studies because it acquires E. chaffeensis, E. canis and A. phagocytophilum infections naturally like humans; both canines and humans are incidental hosts for the tick-borne diseases. (Note: project 1 goals will no longer be pursed because the goals of project 5 will supersede the planned experiments under project 1). Moreover tick transmission studies can be done in this animal model similar to those likely occurring naturally in this host species. Dogs develop persistent infections with all three pathogens. Clinical signs with the infections in the canine host are minor. The Beagle breed is chosen for the studies because it is the most commonly reported breed for similar studies in the literature and moreover, it is easy to work with this breed. Finally, this dog breed is commercially available for use in experimental studies.

   Project 3) RMSF pathogen, Rickettsia rickettsii, causes infections in dogs and people naturally from infected Ixodid (hard) ticks. We previously demonstrated that dogs develop severe form of the RMSF in the canine host (Beagle breed) and that the WCAV confers complete protection against the infection challenge. Canine model is an ideal host for defining various aspects, including assessing host-vector-pathogen interactions and vaccine potential. The beagle is chosen for this study because it is the most commonly reported breed for similar studies in the literature and moreover, it is easy to work with this breed. Finally, this breed of dog is commercially available for use in experimental studies.

   Project 4) Cattle are known to acquire Ehrlichia ruminantium infections naturally in endemic regions. Thus, they are highly susceptible to Heartwater disease and is ideally suited to define if the disease can be a risk for the US cattle industry.

   Project 6) Bovine anaplasmosis is the disease primarily of cattle and is responsible for causing the major economically important disease. Considering the lack of appropriate vaccine preventing the disease and its economical importance coupled with the lack of non-animal alternative to test the vaccine, using steers for the study is necessary. We opted to use steers of the age over 18 months because the disease severity is going to be high for this age group.

3. Justify Animal Numbers

   Justify numbers of animals to be used (attach timeline or flow chart and power analysis, if possible, to describe study groups). This section should include a description of animals used for colony maintenance (breeders and all offspring produced) as well as a description of experimental animal numbers. Total numbers should match the requested numbers in the species section.

   - Animal Numbers Justification
   - The Logical Determination of "N" in Animal Experimentation

- Non-Statistical Approach for Calculating the Optimum Number of Animals Needed in Research
- Statistics and the Issue of Animal Numbers in Research
- JUSTIFY ANIMAL NUMBERS EXAMPLE

Sample size calculation was performed to identify necessary sample size to distinguish between treatment groups accounting for repeated measures over time. Type 1 error at 5% and type 2 error rate set at 20% (80% power). Calculations were performed for differences in percent of T-cells producing interferon, PCR positives assessed by conventional and real time PCR assays, and to measure antibody levels. The largest sample size required was to detect differences requiring 6 dogs in each group to detect the expected differences in pathogenesis, pathogen persistence monitoring, and to differentiate between vaccinated animals and non-vaccinated controls over time. We also will include both sexes to account for variations resulting from sex as a variable. If an experiment is repeated multiple times, then the number of animals will be reduced to account for prior data as the way of justifying the reduced numbers; more details provided in the experimental design section.

6. Animal Husbandry

1. Facilities

In which animal facility will animals be housed?

	Facility
1	[redacted]
2	[redacted]

2. Housing Outside of Facility

Will animals be housed anywhere other than a designated animal housing facility for more than 12 hours (e.g., a laboratory)?

○ Yes ● No

3. Transportation Between Animal Housing/Use Facilities

Will animals be transported with a private vehicle between animal housing/use facilities?

○ Yes ● No

4. Non-Standard Husbandry

A. Does this protocol contain any Prolonged Physical Restraint?

See: ACUC Physical Restraint policy

○ Yes ● No

B. Does this protocol contain any Food/Fluid Regulation?

See: ACUC Food and Fluid Restriction policy

○ Yes
● No
○ Overnight only

C. Does this protocol contain Multiple Survival Surgical Procedures?

See: ACUC Multiple Survival Surgical Procedures policy

○ Yes ● No

D. Does this protocol contain any of the following Non Standard Husbandry?

- ☑ Single housing of social species
- ☐ Wire-bottom cages
- ☐ Special diet/water
- ☐ Extended time to weaning
- ☐ Extended time between cage changes
- ☐ Alternative light cycles
- ☐ Out of range temperatures
- ☐ Cage-size exceptions
- ☐ Other

i. Explain non-standard husbandry and list the length of time the animal will undergo non-standard husbandry.

When performing tick infestation studies, animals will need to be individually housed in their own pens, but at close proximity to each other. This will be important to minimize the damage to the tick cells placed on animals, while not adversely impacting the socialization of animals. Typically, tick cells will be on the animals up to about 7-10 days.

7. Description of Non-Surgical Procedures

1. Sample Collection

Will samples, such as blood or tissues, be collected from live animals? (Include sampling for genotyping.)

● Yes ○ No

A. Sample Type

Type of sample(s):

Mostly blood samples will be collected. In the event of animals requiring termination, such as in the RMSF and in heartwater disease infection studies (projects 3 and 4), tissue samples will be collected from several sources to define gross lesions, histopathological assessments and to look for the presence of pathogen by molecular or cell culture methods. These details were included in the project description.

B. Sample Volume

Volume of sample(s):

Sample volumes will be variable which vary from 1 ml to 20 ml. We provided additional details in the scientific project description section.

C. Sampling Frequency and Duration

Frequency of collection and for how long:

Maximum of 20 ml blood sampling occurs at times and when this happens it will only a once a week. Some times the blood volumes are 10 ml per draw and twice a week. Many times, 1 ml blood will be sampled. These volumes will be similar for dog and cattle studies we proposed. We do not anticipate drawing more than 40 ml of blood a week per animal.

D. Sampling Method

Method of collection:

Blood samples will be collected typically from jugular veins of dogs and cattle. We will also be sampling from cephalic and saphenous veins at times. The blood collections will not be carried out via intracardiac stick.

2. Induced or Spontaneous Neoplasia

Will induced or spontaneous neoplasia occur in live animals?

○ Yes ● No

3. Non-Surgical Procedures

	Procedure	Description of procedure	Building name	Room number or area
1	Tick transmission challenge in dogs	Infection challenge with tick transmission will be done as per our published protocol. Twenty-five adult infected tick pairs (25 males and 25 females) will be allowed to transmission feed on vaccinated dogs for 7 days. Engorged nymphs (obtained from a commercially available source) will be infected with E. chaffeensis, E. canis or A. phagocytophilum by needle inoculation and allowed to molt to the adult stage (Cheng et al. 2015 and Jaworski et al., 2016). To prepare for a tick transmission experiment, we will prepare a tick containment cell for each dog. In our system, we will use containment chambers constructed from the tops of Nalgene jars that are each fitted with a screen and polyvinyl gasket that will be directly glued (3M Scotch-Weld 4799 adhesive) to the shorn back of a dog. Dogs are manually held for the application of the tick containment cell. The shaved area will be approximately 4 inches in diameter and to either the right or left side of the dog over the midback area. The placement of containers will be done 24 hours prior to tick infestation. In addition, the dogs will be fitted with a collar to restrict grooming near the containment chamber. Tick infestations will be accomplished by placing 25 female and 25 male ticks on each dog. We will count ticks to be used for each dog carefully. The transfer of ticks to dogs will be performed by unscrewing the screened top of the container and placing the ticks on the dog. The top of the chamber will be re-secured immediately, and dogs will be returned to individual housing. The dog will be restricted from group play during the 7-day period that the tick containment cells are present. Dogs, tick containment chambers and tick attachments will be monitored daily until all ticks are removed from dogs. Extreme care will be taken, and all ticks will be counted (live or dead) when partially fed ticks are removed on day 7. The Nalgene top of the container will be removed from the polyvinyl gasket and the gasket will be removed by shaving. The dogs will be monitored for an additional four weeks.	[redacted]	to be decided
2	Tick feeding experiments with cattle	Acquisition feeding of ticks for both experiments 1 and 2: To determine if E. ruminantium can be acquired by A. maculatum, nymphs will be allowed to feed on all four groups of animals when we begin to see clinical signs or between 7 to 14 days post infection challenges. Ticks will be allowed to attach for feeding on steers (about 500 naïve nymphal ticks). Ticks will be allowed to secure complete blood meals and then allowed to molt to adult stages. Infection rates in the molted ticks will then be assessed by nested PCR analysis. During tick feeding, animals will be housed separately in pens as necessary and as per the CMG recommendation. Tick cells will be placed on steers. For these experiments, the backs of the animals will be shaved with veterinary clippers. A stockinette sleeve or hard capsule (cell) will be glued to the backs of steers. The firm attachment will be verified after about 24 h and prior to allowing ticks to feed. The cell will remain attached for several weeks. We will monitor twice daily for the retainment of the cell on the animals, as well as its firm attachment. To perform the tick infestation, ticks will be placed inside the cells and closed with the rubber bands or screw cap lid. Ticks will be collected following opening of the cell. We will try to account for all ticks on each animal by counting live and dead ticks. The use of ticks for generating A. marginale infected ticks and their use in infection studies for the bovine anaplasmosis vaccine project were described in the methods section.	[redacted]	to be decided

8. Substances Used in Animals

1. Substances Used in Animals

List the substances you will give the animals here (including vehicles given to controls, hazards, radiation, etc.):

	Substance	Amount/Dose/ Volume	Route	Frequency/ Duration	Hazard	Pharmaceutical Grade
1	Diphenhydramine	1mg per pound	oral	once before I.V. infections or vaccinations	No	Yes
2	Adjuvants	2.5% Montanide™ Gel	subcutaneous	twice	No	Yes
3	In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species	variable	I.V.	once	Yes	No
4	Naive and rickettsial bacteria infected ticks	25 pairs of adults of both sexes or 250 nymphs (for dogs) or 500 nymphs (cattle)	on the shaved surface of the skin	once	Yes	No
5	QS-21 saponin	1 mg	subcutaneous	twice	No	Yes
6	aluminium hydroxide	2%	subcutaneous	twice	No	Yes

2. Non-Pharmaceutical Grade Substances

For those substances that are marked "no" as pharmaceutical grade, list a justification in the space below. Also, include instructions for how they will be mixed to maintain sterility and adjust pH.

3. In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species used for infection studies will be obtained from our laboratory and are always grown in sterile culture conditions. Further, all procedures involving recovering the cultures will also be carried out using sterile experimental conditions.

4. Ticks are natural ectoparasites of animals. We will purchase them from a well-established tick rearing laboratory or maintained by us in the laboratory. It is not possible to obtain pharmacological grade ticks.

3. Substances Used in Animals Personal Protective Equipment (PPE)

PPE is needed to safely handle most materials in the laboratory. In general, a minimum of gloves and lab coat should be used. Other substances would require more PPE such as eye protection, respiratory protection, fume hood, etc. Please notify laboratory members if there are any special precautions that need to be taken when working with the above substances.

Describe the PPE required to handle these substances. You may group substances (e.g., "All substances" or "non-hazardous substances") if all or some use the same PPE. Please list any substances needing alternative or additional PPE separately. You do not have to include additional PPE needed for work with hazards as that will be described in the Hazards section, however, you may include here as well if you wish.

	Substance	Gloves	Eye Protection	Lab Coat	Face Mask	Fume hood	Biosafety cabinet	Double-Gloves	Other	Other PPE
1	In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species	☑	☐	☑	☐	☐	☐	☑	☐	
2	Naive and rickettsial bacteria infected ticks	☑	☐	☑	☐	☐	☐	☑	☐	

Hazardous Agent

If you marked "yes" under Hazard, please complete the "Hazardous Materials" Section that follows.

9. Hazardous Materials

1. Will you use any Biological Hazards?

● Yes ○ No

A. Biological Hazard

List all biological hazards that will be used in live animal work.

	Agent or type of hazard	Donor species	Receiving species	Dose	Route/ Volume of Admin.	Frequency of Admin.	Other
1	In vitro cultures of Ehrlichia, Anaplasma and Rickettsia species	N/A	Canine and bovine	2-5X10^8 organisms/ mL	I.V.	Once	
2	Naive and rickettsial bacteria infected ticks	N/A	Canine and bovine	1-2X10^5 organisms/ mL	N/A	Once	

B. IBC Protocol Number (if applicable for recombinant DNA or biological materials)

List your IBC Approval Number or attach your current IBC application. (Include attachments in the attached files section.)

IBC application will be submitted this week to include the bovine anaplasmosis vaccine project involving steers. This project will be similar to Ehrlichia ruminantium project #4.

☐ Unsubmitted

☑ Submitted

☑ Approved

i. Expiration date

As the protocol is still valit for little over one year, we will very soon submit a request for extension beyond the current expiration deadline of 3/17/2026. Thank you.

C. Biological Hazard - Anticipated Effect(s)

List any anticipated effect(s) of biological hazards on animal.

In project 1 (it will be replaced with project 5), E. chaffeensis random mutant organisms will be used to infect dogs.

In project 2, modified live attenuated vaccine (MLAV) of E. chaffeensis and similarly, E. canis and A. phagocytophilum MLAV will be used for testing the vaccine efficacies. Infection challenges will be performed with in vitro cultured live organisms or using infected ticks. All three pathogens cause only mild disease as detailed in the project description section.

In project 3, rickettsia rickettsii cultured organisms will be used for the infection experiments before or after vaccinations. Non-vaccinated and the pathogen infected animals will develop a severe disease which can be fatal. A severe form of the disease requires close monitoring and observation and guidance of a veterinarian. We expect vaccinated animals to be healthy.

In project 4, cattle will be infected with Ehrlichia ruminantium. The pathogen may or may not cause severe disease, although we will anticipate the likelihood of developing severe clinical signs.

In project 6, cattle will be used to infect with A. marginale. MLAV will be used for testing the vaccine efficacy. Infection challenges will be performed with blood stabilates or using infected ticks. The pathogen can cause moderate to severe disease due to sever loss of RBCs similar to our recent published reports.

In all projects, we will work closely with an attending veterinarian to ensure that animals are cared humanely.

D. Biological Hazard - Housing/Procedure Sites

Where do you anticipate housing/working with animals receiving hazardous or potentially hazardous biological agents? Coordinate with the facility manager then list building and room numbers below.

	Agent	Receiving species	Building	Room or Area	Housing	Procedure
1	Tick transmission of Ehrlichia ruminantium	bovine	[redacted]	Housing and procedures	☑	☑
2	I.V. and tick transmission infections of Ehrlichia, Anaplasma and Rickettsia species	canine	[redacted]	Housing and procedures	☑	☑

E. Biological Hazard - Animal Identification

Explain how animals treated with a biological hazard will be identified (ex. cage card, ear tag, etc.)

- ☐ Cage Card
- ☐ Chip
- ☑ Door Sign

☐ Other

F. Hazardous Agents or By-Products /Presence

The biological hazard or by-products may be present in which of the following?

☐ None
☐ Feces/Urine/Bedding
☐ Saliva
☑ Blood
☐ Aerosols
☐ Animal bite/scratch
☑ Animal carcasses/tissues
☐ Surgical site wound or sore
☐ Other

G. Biological Hazard - Personal Protection Equipment (PPE) and Engineering Controls

PPE to be worn when handling biological hazards. LIDR ABSL-3 includes protective suit, shoe covers, double gloves, full-face PAPR.

	Biological Hazard	Gloves	Eye Protection	Lab Coat	Double-Gloves	Face Mask	Biosafety cabinet	LIDR ABSL-3	Other	Other PPE
1	Naive and rickettsial bacteria infected ticks	☑	☑	☑	☑	☐	☑	☐	☐	

H. Additional Information

List additional information, i.e., special precautions for pregnant women, immunocompromised individuals, special handling, or storage, etc.

2. Will you use any Chemical Hazards?

○ Yes ● No

3. Will you use any Radiation Hazards?

○ Yes ● No

10. Anesthetic Procedures, Pain Control, Other Clinical Drugs

1. Anesthetics, Preanesthetics & Tranquilizers

Will any anesthetics, preanesthetics, or tranquilizers be used?

○ Yes ● No

2. Pharmaceutical Analgesia

○ Yes ● No

3. Non-pharmacologic control of pain

○ Yes ● No

4. Paralytic Agents

○ Yes ● No

5. Antibiotics and Other Agents

(Include any emergency drugs, fluids, etc. here)

● Yes ○ No

6. Antibiotics and Other Agents

List other agents such as antibiotics and other emergency drugs

	Species	Agent	Dose/Volume	Route	Frequency of Admin.
1	Dog	Doxycyclin	10 mg/kg	oral	once per day for four weeks

11. Description of Surgical Procedures

1. Surgical Procedures

Will there be any surgical procedures?

○ Yes ● No

12. Potential Pain or Physical Stress

Potential Pain and/or Distress

Note: Animal Welfare Act regulations define a painful procedure as "any procedure that would reasonably be expected to cause more than slight or momentary pain ... in a human being to which that procedure was applied, that is, pain in excess of that caused by injections or other minor procedures." Procedures reasonably expected to cause pain in the absence of anesthetics or pain relieving drugs should be considered to have the potential to cause pain even with the use of such drugs.

1. Potential Side-Effects and Adverse Health Effects

Describe any potential side-effects or anticipated adverse health effects of all procedures listed in the preceding sections: animal husbandry, description of non-surgical procedures, anesthetic procedures, and surgical procedures.

In projects 2 and 5, clinical signs following infection challenges with Ehrlichia chaffeensis, Ehrlichia canis or Anaplasma phagocytophilum typically include only mild fever (rise in only up to 1.5 C above body temperature). Although lethargy and joint pain are possible, based on our past research experience, we do not anticipate seeing these signs with the infections.

Clinical signs of RMSF in dogs (project 3) may include fever, nausea, vomiting, muscle pain, lack of appetite, edema, and rashes. The disease can progress rapidly to a life-threatening illness within two weeks in naive animals.

Clinical signs of Heartwater disease in cattle resulting from Ehrlichia ruminantium (project 4) may result in significant morbidity. A sudden rise in high fever (107° F) coupled with the loss of appetite, depression and increased respiratory rate are likely. Neurological disorders may follow the respiratory signs which may include excessive chewing movements, incoordination, head tilting, rigid posture and staggered walking with a high-stepping gait. Animals may also exhibit convulsions or be unable to get up. These nervous signs may progress to mortality within one to two days. It is also possible that the animals may not exhibit any nervous signs before progressing to life threatening illness.

Adjuvants in project 3 might possibly induce a reaction. We will closely monitor the animals for such reactions and will follow the guidance of a clinical veterinarian.

2. Assurance of Limited Discomfort and Pain

Describe how it is assured that discomfort and pain are limited to that which is unavoidable for the conduct of this experimentation.

Projects 2 and 5: Ehrlichia and Anaplasma species infections in dogs animals will be observed twice daily with once daily monitoring the body temperatures. Although we do not anticipate serious clinical signs in this study, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer.

Project 3: After Rickettsia rickettsii infection with I.V. and tick transmission following vaccinations and in control groups, animals will be monitored twice daily with once daily monitoring the body temperatures. While we do not anticipate serious clinical signs for the vaccinated groups, all non-vaccinated infection controls are expected to develop a severe clinical disease. Onset of signs for I.V. may occur within three days while tick transmission may take about a week. The clinical signs will include high fever, edema, lethargy and lack of appetite. We will closely monitor the animals' health and promptly communicate with the attending veterinarian for appropriate action particularly if exhibiting depression, lethargy for more than 24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer. Infection control group animals developing severe disease will be requiring euthanasia to alleviate the pain and suffering. We will be following the guidance of the veterinarian regarding when this decision needs to be made. In the event, the animals will be euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) using a commercial euthanasia solution.

Project 4: Ehrlichia ruminantium infections in cattle will be observed twice daily with once daily monitoring the body temperatures. Upon the onset of symptoms, daily collections of 2 ml blood will be initiated. An attending veterinarian will be consulted for appropriate action if the animals appear seriously ill, such as exhibiting depression, lethargy for more than24 hours, changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer, increased heart rate of respiration, or any neurological symptoms. While it is unclear if cattle develop a severe disease with E. ruminantium, in the event we do observe cattle infected with the pathogen develop severe disease, they will be requiring euthanasia to alleviate the pain and suffering. We will be following the guidance of the veterinarian regarding when this decision needs to be made. In the event, such cattle will be euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) by captive bolt method.

Projects 6: Anaplasma marginale infections in cattle will be observed twice daily with once daily monitoring the body temperatures. We anticipate moderate to serious clinical signs in this study. Further, a possibility of animals developing an unrelated illness cannot be ruled out. In such instances, an attending veterinarian will be consulted for appropriate action particularly if exhibiting depression, lethargy for more than24 hours, and/or changes in appetite lasting also for more than 24 hours, fever above 104°F for two days or longer.

3. Pain and Distress Form

Is there a Pain and Distress form associated with this protocol?

See: Painful or Distressful Procedures

● Yes ○ No

Please attach the form in the attachments section of this protocol.

A. Which experimental groups, procedures, or animals require the Pain and Distress form?

Project 3 involving non-vaccinated dogs receiving infection by needle infection and tick transmitted challenge with Rickettsia rickettsii.

Project 4 involving the assessment of parthenogenesis in cattle following infection with Ehrlichia ruminantium.

Note: Files were attached with the previous submission.

13. Disposition of Animals

1. Animal Disposition

Check all that apply

- ☑ Adoption (See MU adoption policy)
- ☐ Market
- ☑ Euthanasia
- ☑ Transfer to different project, PI, or another institution
- ☐ Returns to breeding colony, herd, source, owner, or wildlife site
- ☐ Other

2. Euthanasia

Euthanasia Statement

As noted in the Guide, "Euthanizing animals is psychologically difficult for some animal care, veterinary, and research personnel, particularly if they perform euthanasia repetitively or are emotionally attached to the animals being euthanized (Arluke 1990; NRC 2008; Rollin 1986; Wolfle 1985). When delegating euthanasia responsibilities, supervisors should be sensitive to this issue."

A. Primary Method of Euthanasia

Methods that do not require ACUC proficiency verification

- ☐ Inhalant agent
- ☐ Physical Method with Anesthesia
- ☑ Noninhalent Pharmaceutical Agent

B. Primary Method of Euthanasia (ACUC proficiency verification required unless performed on rodents <7 days old)

Methods requiring ACUC proficiency verification

- [x] Cervical Dislocation without Anesthesia
- [ ] Decapitation without Anesthesia

i. Personnel to be verified for procedure

C. Euthanasia Descriptions

	Species	Agent/Method	Dose/Volume	Route
1	Dog	Euthanasia will be performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA).	Fatal-Plus®, of volume 0.22 ml/kg (86 mg/kg of pentobarbital) will be administered.	I.V. injection
2	Cattle	Captive bolt method	N/A	stunner fires a retractable bolt against the animal's head, primarily into the animal's brain

D. Additional Explanation of Euthanasia Procedures

Include any additional explanation of euthanasia procedures here.

Animals will be checked for the lack of heart beat and breathing to confirm the euthanasia procedure worked accordingly.

E. Scientific Justification for Use

- [x] AVMA Approved Method
- [ ] Not AVMA Approved Method

F. Secondary (Physical) Means of Assuring Euthanasia

- [ ] Bilateral pneumothorax
- [ ] Cervical dislocation
- [ ] Decapitation
- [ ] Exsanguination
- [x] Removal of vital organs

☐ Other

14. Project Information

1.

Associate	Role	Responsibilities	Animal Care & Use	OHSP Training	P&D Training	Survival Surgery
Ganta, Roman Reddy [redacted]	Principal Investigator Authorized to order animals Access to view cages Editor		☑ Feb 2, 2023	☑ Jan 27, 2023	⊖	☑ Feb 2, 2023
[redacted]	Co-Investigator Authorized to order animals Access to view cages Editor	Euthanasia P&D assessment	☑ Jan 27, 2023	☑ Jan 11, 2023	☑ Jan 23, 2023	☑ Jan 19, 2023
[redacted]	Co-Investigator Authorized to order animals Access to view cages Editor	Surgery Euthanasia P&D assessment	☑ Jan 19, 2023	☑ Jan 18, 2023	☑ Jan 23, 2023	☑ Jan 19, 2023
[redacted]	Co-Investigator Authorized to order animals Access to view cages Editor		☑ Jan 20, 2023	☑ Jan 19, 2023	☑ Jan 23, 2023	☑ Jan 20, 2023
[redacted]	Co-Investigator Editor		☑ Feb 2, 2023	☑ Jan 25, 2023	⊖	☑ Feb 2, 2023
[redacted]	Key Personnel		☑ Jan 10, 2025	☑ Jan 9, 2025	⊖	☑ Jan 10, 2025
[redacted]	Key Personnel		☑ Feb 1, 2023	☑ Feb 1, 2023	⊖	☑ Feb 1, 2023
[redacted]	Key Personnel		☑ Apr 23, 2024	☑ Apr 24, 2024	⊖	⊖
[redacted]	Key Personnel		☑ Jan 4, 2024	☑ Aug 28, 2024	⊖	☑ Jan 4, 2024
[redacted]	Key Personnel Access to view cages Lab contact		☑ Apr 26, 2024	☑ Apr 26, 2024	⊖	☑ Apr 26, 2024
[redacted]	Key Personnel		☑ Jan 18, 2023	☑ Jan 18, 2023	☑ Jan 23, 2023	☑ Jan 20, 2023

2. Training and Qualifications

Provide a description of the training and qualifications for each individual listed above under Protocol Associates. Provide adequate detail to allow the ACUC to determine if the individual has adequate training and experience with the species and procedures to perform their role proficiently. If they do not have prior training or experience, how will this be obtained?

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
1	Ganta, Roman Reddy	dogs, sheep, and cattle	Handling, bleeding, vaccine and tick experiments, and measuring temperature.	10 years with dog work in all listed procedures Four months of working with sheep for handling and bleeding, I.V. infections Two months of working with cattle; support help with animal handling	Full-time employee
2	[redacted]	Cattle, sheep, rabbits, barnyard fowls, and wildlife animals	Animal husbandry handling, blood sampling, temperature measurements, surgical procedures, vaccine and tick studies, and euthanasia.	Served as a registered veterinary technician [redacted] 2017– 2022 Animal husbandry (etc) 10+ years Veterinary practice (technician) work with , small, exotic, and wildlife animals 3 years Trapping, hunting, and wildlife management on rural farm 10+ years. Cattle in research – 2 years Sheep in research – 1 year Dogs in research – 2 years Mice in research – 1 year Surgical experience (veterinary practice) many species – 3 years Tick and vaccine studies with animals; dogs, sheep and cattle - about 6 months with each species Euthanasia for two years.	Grad student/ Professional student
3	[redacted]	Cattle and swine	Cattle; Less than a year of experience, collecting blood, performing routine health checks Swine; Less than a year of experience, Collecting blood, taking temperature, weighing, performing routine health checks	Three months each for all the listed procedures	Grad student/ Professional student
4	[redacted]	Cattle, sheep and mice	Cattle: temperature measurement, report clinical signs, help collecting blood samples Sheep: handling, bleeding, temperature measurements Mouse: handling, mice mating, dissection, Peritoneal injection, bleeding (terminal blood collection : cardiac puncture), collect of organs, euthanize using carbon dioxide chamber	Cattle 2 years Sheep; 6 weeks Mouse 4 years	Grad student/ Professional student

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
5	[redacted]	cattle, sheep, goat, dogs, cats, donkeys, horses, and pigs	Animal handling, blood and tissue sample collection, animal health monitoring, surgeries, euthanasia and necropsies.	As a trained veterinarian (DVM equivalent) and also worked in clinical practice with 10 years of experience on all listed procedures	Postdoc fellow/ Resident
6	[redacted]	dogs, sheep, and cattle	Animal handling, blood sample collection, tick studies, and animal health monitoring	Dogs; 8 years of experience with all the above procedures. Cattle; 2 years for the listed procedures Sheep; 2 years also for the above listed procedures	Full-time employee
7	[redacted]	3 years with mice, six months each with sheep and rats.	Assists with animal handling and bleeding.	She doesn't have prior experience with dogs, but has experience with mice and sheep. She will be trained by one of our group members having high level experience prior to her helping with the projects.	Full-time employee
8	[redacted]	She doesn't have prior experience with large animals, although she worked with frogs for three years. She will be trained by one of our group members having high level experience prior to her helping with the projects.	Bleeding and handling	As she doesn't have a project-specific experience, she will be trained by one of our group members having high level experience prior to her helping with the projects prior to working with dogs and cattle.	Full-time employee
9	[redacted]	She has about 7 years of experience working with mice with handling, bleeding, and injections etc. She will be trained by one of our group members having high level experience prior to her helping with the projects when using large animals.	Handling and bleeding	She has about 7 years of experience working with mice with handling, bleeding, and injections. As she doesn't have a project-specific experience, she will be trained by one of our group members having high level experience prior to her helping with the projects prior to working with dogs and cattle.	Full-time employee
10	[redacted]	None; she will be trained by one of our experienced team members.	handling and bleeding	she will be trained by our experienced team members.	Full-time employee

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
11	[redacted]	mice and Chinchilla	handling and bleeding.	He will be trained by our trained staff.	Full-time employee

Training Requirements

Note: The ACUC required Basic Training can be found at: https://research.missouri.edu/acqa/. This training must be updated every three years in order to receive protocol approval.

Note: It is the Principal Investigator's responsibility to ensure that all persons listed in Protocol Associates above participate in the MU Occupational Health and Safety Program. See Section 7:020 MU Business Policy and Procedures Manual for details. For enrollment procedures visit the OHSP website.

3. Funding Source

What is the funding source for this project? (Note: If funded internally or by a non-peer-reviewing agency, a peer review of scientific merit may be required.)

- [x] PHS (NIH, CDC, FDA, NSF, NASA)
- [ ] DoD
- [ ] VA
- [ ] AHA
- [x] USDA
- [ ] Foundation/Industry
- [ ] Internal
- [ ] Other

15. Refinements or Literature Search

Attach relevant files in the attached files section.

1. Painful Procedures

Any procedure that may potentially cause more than momentary or slight pain or distress requires a literature search for animal alternatives.

Are you performing any procedures that may potentially cause more than momentary or slight pain or distress?

● Yes ○ No

2. USDA Covered Species

Does this protocol utilize animals covered by the Animal Welfare Act or assigned to Category E? (AWA covered species include all warm blooded animals except birds, rats of the genus Rattus, and mice of the genus Mus, bred for use in research, horses not used for research purposes, and other farm animals.)

● Yes, includes USDA covered species or Category E ○ No

3. Includes USDA covered species or Category E

Search for Animal Alternatives

In the literature search and in the written narrative, replacement by non-animal systems, reduction in numbers of animals and refinement of experimental methods (the three R's) must be addressed.

Provide at least two sources of information: one of these sources must be a scientific literature database; documented expert consultation may be used as one source of information.

If you are in the School of Medicine and need assistance with this item, please contact Rachel Alexander, HSL Research Support Librarian, at AlexanderRL@health.missouri.edu. Others can contact the Zalk Veterinary Medical Library, at MU CVM VetMed Library for help.

See also:

https://www.nal.usda.gov/awic/sample-searches
https://library.missouri.edu
Literature Search Help

A. Source 1: Literature Database

Complete the information below:

	Date of Search	Name of Database	Years Covered by Search	Keywords and Search Strategy
1	January 8, 2025	Pubmed	1950 to current	For project 1) Searched Ehrlichia chaffeensis AND mutagenesis AND pathogenesis with or without the word dog For project 2) The following words in several combinations were searched; vaccine OR vaccines OR attenuated live vaccines AND Anaplasma AND Ehrlichia AND dogs For project 3) vaccine OR vaccines OR attenuated live vaccine OR attenuated live vaccines AND dog OR dogs OR canine AND Rickettsia OR Rocky Mountain spotted fever OR Rickettsia rickettsii AND Rocky Mountain spotted fever vaccine For project 4) Searched the following combinations and other variations of the words; (((Salivary Glands) OR (Salivary Gland)) OR (saliva)) AND ((((((heartwater) OR (heartwater disease)) OR (ehrlichia ruminantium)) OR (cowdria ruminantium)) AND (((cattle) OR (ruminant)) OR (ruminants))) AND ((((((amblyomma) OR (amblyomma maculatum)) OR (Gulf coast tick)) OR (gulf coast ticks)) OR (tick, gulf coast)) OR (ticks, gulf coast))) For project 6) searched using the following word combination: vaccine OR vaccines OR attenuated live vaccines AND bovine anaplasmosis AND anaplasma marginale AND cattle
2	April 28, 2024	Pubmed	1950-current	For project 5) Searched Ehrlichia chaffeensis AND mutagenesis AND pathogenesis with or without the word dog

B. Source 2: Literature Database

For the second source you may use a literature database search or an expert consultation (see following question).

	Date of Search	Name of Database	Years Covered by Search	Keywords and Search Strategy
1	Janyary 8, 2025	CAB Direct	1920 to present	For project 1) Searched Ehrlichia chaffeensis AND mutagenesis AND pathogenesis with or without the word dog For project 2) The following words in several combinations were searched; vaccine OR vaccines OR attenuated live vaccines AND Anaplasma AND Ehrlichia AND dogs For project 3) vaccine OR vaccines OR attenuated live vaccine OR attenuated live vaccines AND dog OR dogs OR canine AND Rickettsia OR Rocky Mountain spotted fever OR Rickettsia rickettsii AND Rocky Mountain spotted fever vaccine For project 4) Searched the following combinations and other variations of the words; (((Salivary Glands) OR (Salivary Gland)) OR (saliva)) AND ((((((heartwater) OR (heartwater disease)) OR (ehrlichia ruminantium)) OR (cowdria ruminantium)) AND (((cattle) OR (ruminant)) OR (ruminants))) AND ((((((amblyomma) OR (amblyomma maculatum)) OR (Gulf coast tick)) OR (gulf coast ticks)) OR (tick, gulf coast)) OR (ticks, gulf coast))) For project 6) the search terms remain the same as for project 2 with minor modifications. The following was the search words combination: vaccine OR vaccines OR attenuated live vaccines AND bovine anaplasmosis AND anaplasma marginale AND cattle

C. Source 2: Expert Consultation (alternative)

For the second source you may use a literature database search or an expert consultation. Documented expert consultation may be used as one source of information.

No Sources...

D. Animal Alternatives Narrative

Based on the information from the sources above, provide a written narrative of alternatives to procedures that may potentially cause more than momentary or slight pain or distress. The narrative should be such that the ACUC can readily assess whether the search topics were appropriate and whether the search was sufficiently thorough.

If a possible alternative was identified or is known, but will not be employed, discuss why.

For project 1 PubMed search yielded 13 citations and 7 of them represent the work we previously published. The remaining 6, included a review, and are unrelated to the work proposed in our study. There is no evidence of duplication of our current work with any published research including our previous research. CAB Direct search with the similar word search yielded only three citations and two of which were our previous articles and a review. Again, we found no evidence for duplication.

For project 2, despite the use of several combinations of the listed words yielding 278 citation on the PubMed search, there was no evidence of any published work reporting any data on similar topics as we planned in the current study. Specifically, description of vaccine development, particularly using the live attenuated versions of Ehrlichia and Anaplasma pathogens impacting people or dogs are non-existing. CAB Direct for a similar search did not result in the detection of published research related to our proposed goals.

For project 3, Pubmed search resulted in 92 articles; 23 of which are related to vaccine studies in the past. Our recent publication on the topic is among the identified publications (Alhassan et al. 2019, Infect Immun. 2019 Jan 24;87(2):e00628-18. doi: 10.1128/IAI.00628-18). This article summarizes all the work prior to our study. Notably, the prior research focused mostly on inactivated vaccines did not translate in

outcomes research for the RMSF vaccine development. The review article Richards [Expert Rev Vaccines. 2004 Oct;3(5):541-55. doi: 10.1586/14760584.3.5.541] is among the articles found. It summarizes the importance of our study as it stated that the vaccine studies in the past century to prevention of rickettsial diseases did not yield any rickettsial vaccines manufactured and/or licenses. Also stated that "Early rickettsial vaccines were difficult, expensive and very hazardous to produce." Based on all these analyses, it is evident that the only significant publication related to vaccine studies is our recent publication. The current project, thus, extends our previous published work in developing vaccine that will likely be valuable for application for controlling the RMSF in dogs and possibly in people in the near future. Cab Direct found four results which included our above listed publication (Alhassan et al. 2019) and the remaining articles are unrelated the proposed project goals. Our prior publication indeed is the basis for expanding research on the current funded NIH grant for which this search was performed.

For project 4, a maximum of 9 citations were identified, but none of the publications were directly relevant to the project description we proposed. Thus, we will not be duplicating any prior studies.

For project 5, PubMed search yielded the same 13 citations and 7 of them represent the work we previously published. The remaining 6, included a review, and are unrelated to the work proposed in our study. There is no evidence of duplication of our current work with any published research including our previous research. CAB Direct search with the similar word search yielded only three citations and two of which were our previous articles and a review. Again, we found no evidence for duplication.

For project 6, Pubmed search yielded 210 results while CAB Direct resulted in 30 citations. Most of the citations are unrelated except for our prior published work related to the development of modified live attenuated vaccine to prevent A. marginale infections in cattle from IV infection and tick transmitted infection. These publications belonged to our prior published research.

16. Investigator Assurances

1. ABSL-2 Assurance

I will provide training to the husbandry/veterinary staff at least 48 hours prior to exposing animals to a biohazard regarding (but not limited to): the health hazards and symptoms of the biohazard(s) being used; husbandry related research specific SOP's (e.g. handling live exposed animals and contaminated cages); and animal/carcass disposition.

- ● Yes, I will meet the requirements of this statement.
- ○ No, I will not meet the requirements of this statement.
- ○ Not Applicable

2. Investigator Assurances

- ☑ 1. The information provided herein is accurate to the best of my knowledge.
- ☑ 2. Procedures involving vertebrate animals will be performed only by trained or experienced personnel, or under the direct supervision of trained or experienced persons.
- ☑ 3. Any change in the care and use of vertebrate animals involved in this protocol, will be promptly forwarded to the MU ACUC for review; such changes will not be implemented until the committee's approval is obtained.
- ☑ 4. The number of animals proposed is the minimum necessary to conduct valid experimentation.
- ☑ 5. I assure that I am not unnecessarily duplicating previous experiments.
- ☑ 6. I have considered alternative methods to using animals.

- [x] 7. I understand that animal housing must be coordinated with the facility veterinarian and/or facility manager and that approval of this protocol does not guarantee space to house animals.

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