











































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	██████████
2	██████████

## 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	<p>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 <i>E. chaffeensis</i>, <i>E. canis</i> or <i>A. phagocytophilum</i> 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.</p>	██████████ ██████	to be decided
2	Tick feeding experiments with cattle	<p>Acquisition feeding of ticks for both experiments 1 and 2: To determine if <i>E. ruminantium</i> can be acquired by <i>A. maculatum</i>, 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.</p>	██████████ ██████	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	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	
2	Naive and rickettsial bacteria infected ticks	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	

**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 <sup>8</sup> organisms/mL	I.V.	Once	
2	Naive and rickettsial bacteria infected ticks	N/A	Canine and bovine	1-2X10 <sup>5</sup> 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 <i>Ehrlichia ruminantium</i>	bovine	██████████ ██████	Housing and procedures	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
2	I.V. and tick transmission infections of <i>Ehrlichia</i> , <i>Anaplasma</i> and <i>Rickettsia</i> species	canine	██████████ ██████	Housing and procedures	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>

**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	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	

**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 than 24 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 than 24 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

- 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. Method of Euthanasia

Select the method of euthanasia

- Inhalant agent
- Physical Method without Anesthesia
- Physical Method with Anesthesia
- 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

- AVMA Approved Method
- Not AVMA Approved Method

E. Secondary (Physical) Means of Assuring Euthanasia

- Bilateral pneumothorax
- Cervical dislocation
- Decapitation
- Exsanguination
- Removal of vital organs



Other

WHITE COAT  
WASTE  
PROJECT

## 14. Project Information

1.

Associate	Role	Responsibilities	OHSP Training	Animal Care & Use	Survival Surgery	P&D Training
Ganta, Roman Reddy ██████████	Principal Investigator Authorized to order animals Access to view cages		☑ Jan 27, 2023	☑ Feb 1, 2023	☑ Feb 2, 2023	☐
██████████ ██████████	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
██████████ ██████████	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
██████████ ██████████	Co-Investigator Authorized to order animals Access to view cages		☑ Jan 19, 2023	☑ Jan 20, 2023	☑ Jan 20, 2023	☑ Jan 23, 2023
██████████ ██████████	Co-Investigator		☑ Jan 25, 2023	☑ Feb 2, 2023	☑ Feb 2, 2023	☐
██████████ ██████████	Co-Investigator Authorized to order animals Access to view cages		☑ Jan 18, 2023	☑ Jan 19, 2023	☑ Jan 26, 2023	☑ Jan 23, 2023
██████████ ██████████	Key Personnel		☑ Feb 1, 2023	☑ Feb 1, 2023	☑ Feb 1, 2023	☐
██████████ ██████████	Key Personnel		☑ Jan 19, 2023	☑ Jan 20, 2023	☐	☐
██████████ ██████████	Key Personnel		☑ Jul 2, 2023	☑ Jul 2, 2023	☐	☐
██████████ ██████████	Key Personnel		☑ Feb 10, 2023	☑ Oct 21, 2020	☐	☐
██████████ ██████████	Key Personnel		☑ Jan 18, 2023	☑ Jan 18, 2023	☑ Jan 20, 2023	☑ Jan 23, 2023
██████████ ██████████	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?

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WHITE COAT  
WASTE  
PROJECT

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 ██████ ██████ ██████	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.	██████ ██████ ██████
3 ██████ ██████	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	██████ ██████ ██████
4 ██████ ██████	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	██████ ██████ ██████

	<b>Associate</b>	<b>Experience with research animals:</b>	<b>Which procedures will this person perform?</b>	<b>Experience with each procedure:</b>	<b>Employment Status</b>
5	██████ ██████	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	██████ ██████
6	██████ ██████	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	██████	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	██████ ██████	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	██████ ██████	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	██████ ██████	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	██████ ██████	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	██████████ ██████ ██████████	None	Assist in holding animals and sample collections	None; she will be trained by one of our experienced team members.	██████████ ██████

**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.)

- PHS (NIH, CDC, FDA, NSF, NASA)
- DoD
- VA
- AHA
- 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:

	<b>Date of Search</b>	<b>Name of Database</b>	<b>Years Covered by Search</b>	<b>Keywords and Search Strategy</b>
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).



	<b>Date of Search</b>	<b>Name of Database</b>	<b>Years Covered by Search</b>	<b>Keywords and Search Strategy</b>
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  $\geq$  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  $\geq$  0.8, contact veterinarian**













**If total score  $\geq$  1.0, euthanize animal unless veterinarian permits a recheck\***

\*If an animal's total score is  $\geq$  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 $\leq$ 1-2 / 9 (very thin, bones can be felt easily and are visible)
Temperature	0.0	<103.5°F
	0.2	$\geq$ 103.5 but <105°F
	0.4	$\geq$ 105 but <106°F
	1.0	$\geq$ 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

# 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.	  <span style="font-size: 2em; font-weight: bold; color: #008080;">1</span>
	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.	  <span style="font-size: 2em; font-weight: bold; color: #804000;">4</span>
	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.	  <span style="font-size: 2em; font-weight: bold; color: #804000;">6</span>
	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.	  <span style="font-size: 2em; font-weight: bold; color: #804000;">7</span>
	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.	  <span style="font-size: 2em; font-weight: bold; color: #804000;">8</span>
	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.	  <span style="font-size: 2em; font-weight: bold; color: #804000;">9</span>

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, Barigas JW, Moyars 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

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

Koaly, 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



# 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  $\geq$  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  $\geq$  0.8 or neurologic signs present, contact veterinarian**  
**If total score  $\geq$  1.0, euthanize animal unless veterinarian permits a recheck\***

\*If an animal's total score is  $\geq$  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	$\geq$ 103.5 but <105°F
	0.4	$\geq$ 105 but <106°F
	1.0	$\geq$ 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)

