#### I. <u>Title:</u> Towards predicting plague epizootics: Understanding free-living amoeba as an interepizootic host for sylvatic plague

<u>Rationale:</u> Yersinia pestis, the causative agent of bubonic plague, is transmitted by flea bite. The plague is difficult to eradicate because it is widespread in wild rodents and their associated fleas, and has a continuing foci of endemicity during quiescence. Since its introduction into North America at the turn of the 20<sup>th</sup> century, plague has caused the precipitous decline of wild rodent species e.g. prairie dogs and brought their predator, the black-footed ferret, to the brink of extinction. Natural sylvatic cycles of plague are composed of two phases: i) a prolonged quiescent phase during which *Y. pestis* persists undetected in an unknown reservoir, and ii) unpredictable re-emergence of an epizootic phase characterized by rampant death of susceptible hosts (prairie dogs, the endangered black-footed ferret) and amplified levels of disease that heighten risk of transmission to other animals (e.g. mountain lions, domestic cats and dogs). Understanding how plague persists during inter-epizootic periods and then re-emerges into epizootics is paramount to improving approaches to understand risk and predict and prevent epizootic events and large scale animal death.

<u>Hypothesis:</u> We hypothesize that bactiverous free-living soil amoeba (FLA) serve as an alternate host and soil reservoir for *Y. pestis* during quiescent inter-epizootic plague cycles that supports reactivation of plague epizootics.

<u>Study Design</u>: In this study we propose to make an experimental investigation into whether FLA that are isolated from an active plague foci can support long term survival and /or replication of *Y. pestis* in controlled laboratory culture. This will be followed by determining if such an interaction is supported in a rodent burrow soil simulating environment under conditions that are consistent with seasonal epizootic and inter-epizootic plague cycles.

<u>Preliminary Data:</u> Our preliminary studies provide the first evidence for replication and prolonged survival of *Y. pestis* within a laboratory strain of the FLA, *Acanthamoeba castellanii*. We have subsequently isolated and identified FLA species, including *Acanthamoeba* spp, from black-tailed prairie dog burrow soils of a plague foci in the Cathy Fromme prairie in Colorado. This prairie dog community has recently experienced a die-off from plague. These data support that colocalization of *Y. pestis* and FLA in prairie dog burrows is probable, and warrants further investigation of this interaction.

<u>Expected Results:</u> We anticipate developing essential method and expertise to detect the *Y. pestis*amoeba interaction in endemic plague foci. As well, this study should advance understanding of how amoeba act as a reservoir host for *Y. pestis* during quiescence. This work has the potential to initiate pioneering efforts into detecting the *Y. pestis*-amoeba interaction in plague endemic areas, and developing strategies to predict plague recrudescence and epizootics.

<u>Budget and Timeline:</u> During Year One the ability of amoeba from plague endemic areas to support long term survival and and/or replication as outlined in Aim 1, will be performed. Concurrently, optimization of experiments in Aim 2 will be undertaken. In Year Two final investigation into the dynamics of bacterial survival in amoeba in a burrow soil simulating environment and manuscript preparation and submission will be accomplished. Funding **Budget and Two** five a very two years is sought.

<u>Potential Impact for Animal Health:</u> This research aims to discover the mechanisms that allow *Y. pestis* inter-epizootic persistence and drive its re-emergence. This is especially significant as current biosurveillance methods are focused on serology testing in wild rodents or bacterial culturing from fleas that often prove unsuccessful at detecting plague even immediately prior to epizootic events. Knowledge of *Y. pestis* survival in plague foci endemic FLA will allow specific detection of *Y. pestis* in plague foci. The approach of predicting a plague outbreak, will allow preemptive measures to be implemented (e.g insecticide spraying to kill fleas in rodent burrows prior to reactivation of an epizootic; insecticide use is usually only undertaken when rodent deaths are observed as indication of the presence of disease). Pre-emptive measures can prevent large scale prairie dog and back-footed ferret die-off and death in the first place. As such the research is consistent with the Morris Animal Foundation funding criteria for relevant research. III. Name, Institution and email address Principal Investigator:

Viveka Vadyvaloo, PhD Assistant Professor



Co-Investigator (graduate student):

Javier Benavides- Montaño, DVM



Technician:

To be determined

#### IV Study proposal

#### Specific, testable Hypothesis and Objectives 1.

Yersinia pestis, the causative agent of sylvatic plaque, is transmitted by flea bite. Natural sylvatic cycles of the plague are composed of two phases: 1] a prolonged quiescent phase during which Y. pestis persists undetected in an unknown reservoir, and 2] unpredictable re-emergence of an epizootic phase characterized by rampant death of susceptible animal hosts and heightened risk of transmission to other animal species including domestic cats and dogs. Understanding how plague persists during interepizootic periods and then re-emerges into epizootics is needed to improve approaches to understand risk and reduce epizootic transmission. Identification of the reservoir that supports long-term guiescent persistence of Y. pestis however remains enigmatic. Epidemiologists/ecologists have postulated that bacterivorous soil FLA phagocytose Y. pestis and serve as a soil reservoir for this pathogen, similar to what has been demonstrated for other bacterial pathogens, e.g. Legionella pneumophila (1-3). This model posits that, in the first step, Y. pestis enters and survives, and remains protected within the vegetative active trophozoite amoeba forms in the soil. In the next step which leads to recrudescence of the epizootic cycle, Y. pestis infected amoeba can act as a vehicle for re-infection of a foraging rodent or larval flea in the soil (Fig 1). As proof-of-concept our preliminary studies provide the first evidence for replication and prolonged survival of Y. pestis as residents of vacuoles in amoeba trophozoite forms of the soil FLA, Acanthamoeba castellanii. The next logical steps in defining if FLA can serve as natural reservoir hosts of quiescent Y. pestis, is to understand the natural biological context of the Y. pestis-



interaction. amoeba Our proposal aims to address this knowledge gap by determining if **FLA** present in burrows of blacktailed prairie dogs that have recently experienced plaque epizootics. are capable of supporting long term survival and replication of Υ. pestis.

**Specific Aim 1:** Determine if FLA present in burrows of black-tailed prairie dogs that have recently experienced plague epizootics are capable of supporting long term

enzootic plague periods

survival and replication of Y. pestis. Abundant and diverse FLA that can serve as reservoirs for bacterial pathogens have been documented in areas endemic for their associated diseases e.g. L. pneumophila (2, 3) and Mycobacterium ulcerans (4). To this end, we have cultivated viable FLA, including Acanthamoeba spp. from soil collected from burrows of prairie dogs that have recently experienced a die-off from plaque. A quantitative determination of long-term survival and replication of Y. pestis in trophozoites will be achieved for the different FLA species. Defining the level of Y. pestis surviving in trophozoites can be used to establish the threshold for detection of infected amoeba in rodent burrow soils of endemic plaque foci

Specific Aim 2: Determine if the ability of Y. pestis to survive long-term and/or replicate in amoeba can be recapitulated in a simulated burrow soil environment. This simulated environment should mimic the environmental conditions during epizootics or the inter-epizootic stage. Thus far we have shown experimentally that under favorable axenic laboratory medium culture conditions, Y. pestis has

the ability to survive and replicate in amoeba (Fig 2). This is the first demonstration that *Y. pestis* is able to evade digestion and survive for a prolonged period of time in bactiverous amoeba. However it is not known if such an interaction is supported in a natural soil environment.

#### 2. Justification , Significance and Literature Review

Impact of plague on wild rodent species and the endangered black-footed ferret. Since the introduction of plague to North America at the turn of the 20th century, Y. pestis has established itself in endemic wild rodent populations (5). This has led to the precipitous decline of particularly susceptible endemic rodent species e.g. prairie dogs (Cynomys spp.). The prairie dog predator, and only endemic US ferret population, the black-footed ferret (Mustela nigripes), has also been brought to the brink of extinction due to plague. To preserve the endangered black-footed ferret, a captive-breeding program aimed at latter reintroduction of this species into viable prairie dog habitat has been ongoing (6). A successful full recovery of the species however has yet to be achieved, and continues to be a challenge due to continued plague outbreaks in these areas. Amoeba as an inter-epizootic reservoir host for Y. pestis: Y. pestis, the etiological agent of plague has arisen as a clonal variant of Yersinia pseudotuberculosis, which can survive for long periods in soil (7), while Y. pestis cannot. Comparative genomics between Y. pestis and Y. pseudotuberculosis (7) has revealed that the Y. pestis genome has undergone genomic decay that has resulted in degradation of several metabolic pathways. This is believed to account for its host (flea or rodent) requirement for propagation. Beyond these two host environments however, there has been minimal evidence for Y. pestis to transmit or persist in other life stages. This is consistent with the long-standing inter-epizootic host persistence theory for Y. pestis, which postulates that plaque is maintained naturally in infected fleas feeding on a heterogeneous

population of susceptible and relatively resistant hosts. This model does not address however the following conundrums that collectively suggest an additional environmental maintenance reservoir for Y. pestis. These are that: (i) bacteremia levels of relatively resistant hosts are low and insufficient for flea acquisition of Y. pestis from the bloodmeal to perpetuate the flea-rodent transmission cycle (8), (ii) seldom are infected fleas or seropositive rodents recovered after surveillance of endemic plague foci (9), (iii) re-emergence and epizootics are infrequent and can sometimes occur >50 years or more, beyond the lifespan of fleas and hosts, (iv) ecological modeling studies show that susceptible burrow rodent metapopulations have low probability of supporting plague guiescence and reemergence to produce epizootics at regular intervals (10). Alternately, a soil reservoir for maintenance of Y. pestis during inter-epizootic periods has also been hypothesized, and under experimental conditions that assume Y. pestis is free-living and metabolically



<u>Fig. 2.</u> **Survival and replication of** *Y. pestis* **strains in trophozoites** of *A. castellanii* at epizootic temperature of 25°C (2h, 24h and 1 week) and inter-epizootic temperature of 4°C (1 week). Error bars represent mean±SD of at least 3 independent biological replicates. Statistical significance from One Way ANOVA analysis followed by a post Tukey's test is represented by \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 Y. *pestis* strains are white bars: KIM5; grey bars:KIM6+; blue bars: *phoP* mutant. nd= not detected. **Electron micrograph images** of uninfected amoeba trophozoites (B) and Y. *pestis* KIM5 infected A. *castellanii* trophozoites. (B) Y. *pestis* KIM5 infected amoeba. (\*) Y. *pestis* KIM5, inset image in C represents intra-amoebal GFP-Y. *pestis* KIM5. V=vacuole, N=nucleus, P=pseudopodia, M= mitochondria

active, persistence of *Y. pestis* in soil has been demonstrated, but only over short periods of time (9, 11-13). This fails to explain limited detection of the organism in soil during inter-epizootic periods at plague foci. <u>Why amoeba?</u> Nevertheless, the survival of *Y. pestis* inside an alternate host, like bactiverous soil FLA, could simultaneously explain the existence of a soil reservoir, and satisfy a host requirement for *Y. pestis* and the ability to persist and maintain plague cycles. Survival of several bacterial pathogens, including *Legionella pneumophila* (1), *Francisella tularensis* (14), and *Mycobacterium avium* (15), has been mechanistically characterized within both the metabolically active trophozoite and dormant cyst forms of the host-like soil FLA *Acanthamoeba* spp. These bacteria are able to survive and are protected from the environment within amoeba (16, 17). To evade being digested by amoeba, similar mechanisms as those used to resist digestion in mammalian host immune cells (e.g. macrophages) are often used by pathogenic bacteria (18). One example, is that of *Legionella pneumophila*, the bacterial agent of a pneumonic infection called Legionnaire's disease that persists in aquatic systems in environmental FLA (18). These bacteria reside in phagosomes and evade phagocytic destruction by deploying bacterial effectors secreted from the Dot/Icm secretion system in both amoeba and macrophages (18). *L. pneumophila* demonstrates enhanced virulence following intracellular persistence in amoeba (1, 19), and intranasal infection of mice with *L. pneumophila*-containing amoeba leads to development of Legionnaire's disease (2, 3).

<u>The prairie burrow environment is conducive to an amoeba-Y. pestis interaction.</u> Black-tailed prairie dogs, *Cynomys ludovicianus*, are ground dwelling rodents that live in large social communities connected by extensive tunnels and burrows (called towns) in the short grass prairie plains in Northern Colorado. These rodents epitomize the sporadic nature of plague because they experience extinctions from plague epizootics frequently during warmer and wetter climatic conditions as opposed to absence of plague during drought conditions (20, 21). During these epizootics the microclimate maintained in the prairie dog burrow is conducive to maintaining amoeba in their trophozoite form e.g. moist soil (22, 23), high organic nutritional content maintained by plant material that rodents feed on, rodent feces, and decaying carcasses of rodents that have succumbed to plague, high bacterial content as a nutritional source for bactiverous amoeba (21, 23). Consistent with this, *Acanthamoeba polyphaga* FLA species is commonly present in soils of the Central experimental station short grass prairie where black-tailed prairie dog



Fig. 3. Electron micrograph images of trophozoites (A, C) and double walled mature cyst forms (B, D) of lab *A. castellanii* and burrow *Acanthamoeba* spp. 13CO0814 respectively. Burrow *Acanthamoeba* has a pronounced hyaloplasma that supports it faster locomotion than *A. castellanii* in lab culture medium. populations have often become extinct from plague (15, 24, 25). *A. polyphaga* acts as an environmental host to various pathogens (26, 27). Based on these factors a model for the survival and recrudescence of *Y. pestis* from an amoeba host has been envisaged. In this model *Y. pestis* enters and survives in vegetative active trophozoite amoeba forms during cooler quiescent periods. In the second step, during favorable warmer and wetter environmental conditions infected trophozoites will be acquired by flea or rodents resulting in reactivation of the epizootic disease cycle (Fig 1).

#### 3. Preliminary data

To address this model we quantified the intracellular survival of bacteria after allowing bacteria to be phagocytosed for an hour and killing of extracellular/unphagocytosed then bacteria with gentamycin. Bacterial survival in trophozoites is then assessed in a nutritive axenic culture medium, PYG, which supports continued metabolic activity in trophozoites. Our preliminary studies consequently provide the first quantitative evidence for entry, prolonged survival and replication of Y. pestis within trophozoites (Fig. 2) of A. castellanii. We used a strain that contained a mutation in PhoP which is a factor that is required for Y. pestis to enter and survive in macrophages (Fig 2) (28, 29). Similar to its role in macrophages, PhoP appears to be required for entry in amoeba. This data is in keeping with the prevailing paradigm that bacteria use conserved mechanisms to survive both in amoeba and macrophages

(18, 30). Consistent with our quantitative data, electron microscopy (EM) shows that intact *Y. pestis* occurs in phagosomes of *A. castellanii* (Fig 2C). A previous study has observed *Y. pestis* within the FLA, *Hartmanella rhysodes* (31). These findings support our model and identifies FLA as an alternate host (besides rodents and fleas) that can support long-term survival and replication of *Y. pestis*.

Most approaches in plague research are aimed towards development of vaccine strategies to prevent plague. Only a few studies focus on plague surveillance and predicting re-emergence as a means to preventing the disease. Plague surveillance in endemic areas is challenging based on the difficulty of detecting the pathogen in the environment during inter-epizootic cycles (32). Indeed a lack of detection of Y. pestis in the soil in endemic areas during inter-epizootic periods may be related to the bacteria surviving within an amoeba host at low levels, rather than freely in the soil. If Y, pestis can be detected during this quiescent cycle, the ability to predict its re-emergence will be practical. This could lead to novel strategies being implemented that prevent disease prior to epizootics. As the biological relevance of Y. pestis being harbored by amoeba has not been established, this proposal aims to establish biological relevance of Y. pestis in FLA that we have identified from burrows of black-tailed prairie dogs that have recently experienced plague epizootics. Therefore in a first approach to determine if Y. pestis can be harbored in FLA species, soil from burrows in the Cathy Fromme short grass prairie in Northern Colorado where plague epizootics resulting in black-tailed prairie dogs die-offs have very recently been recorded, was investigated for the presence of FLA. We received soil sample collections from our collaborators (Drs Eisen and Montenieri) of the Plaque Group at the CDC in Fort Collins, CO. Following standard isolation and enrichment culturing methods, and characterization based on morphology and 18S rRNA sequencing, we have been able to culture a few FLA species including Hartmanella and Acanthamoeba spp., and have achieved culturing a pure isolate of the Acanthamoeba spp. (Fig. 3).

#### 4. Experimental methods and design

The Y. pestis KIM5 (33) and isogenic KIM5 phoP mutant (28) avirulent strains will be used for the studies outlined below. The Y. pestis KIM5 strain contains the T3SS and is genetically more representative of a fully virulent Y. pestis strain. Because the Y. pestis phoP mutant strain (34) is defective in its ability to productively infect A. castellanii (Fig. 2) it will be used as a negative control in quantitative intracellular survival studies. The trophozoite life stage is predicted to support infection and re-emergence.

**Specific Aim 1:** Determine if ecologically relevant burrow Acanthamoeba spp. can support intracellular survival and replication of Y. pestis

**Rationale:** Y. pestis is expected to enter the soil in rodent burrows in a plague endemic area, from a decomposing carcass of a rodent that has succumbed to plague, or from flea feces (Fig 1). Within soil numerous FLA species are present that likely phagocytose Y. pestis. Within amoeba Y. pestis is protected from the harsh soil environment (Fig 1). Acanthamoeba spp trophozoites have a growing range of 4-27°C (35, 36). This is consistent with extreme lower and upper temperature limits expected in subterranean rodent burrows during seasonal inter-epizootic and epizootic periods (23, 37). These temperature limits will be used to test long term intracellular survival of Y. pestis. Similar to A. castellanii we have observed that at 4°C temperature, amoeba remain as trophozoites for over a month at least, and do not multiply as rapidly as at ambient temperature, consistent with published findings.

**Methodology**: Gentamycin protection assays designed to evaluate Y. pestis survival and replication will be used to quantify Y. pestis survival and replication in naturally occurring FLA. Bacteria will be grown at 25°C consistent with the burrow temperature during epizootics prior to co-culture. Co-cultures will be incubated at both ambient temperature of 25°C and 4°C to investigate the intracellular survival and/or replication at expected epizootic and inter-epizootic periods, respectively. The assay is a modification of our published methods used to determine that Y. pseudotuberculosis, and not Escherichia coli DH5a survives and replicates in A. castellanii trophozoites and cysts (38). A. castellanii will be used as a positive control for its ability to phagocytose Y. pestis. Klebsiella pneumoniae will be used as a negative control as it is utilized as a food source by burrow FLA. At least 3 independent biological replicates of interaction assays will be undertaken and a Student's T-test will be undertaken to determine significant differences in entry/uptake and efficiency of survival and replication in natural burrow amoeba Acanthamoeba for the two Y. pestis strains to be tested. Analysis and alternatives: Y. pestis is expected to survive and replicate in burrow amoeba at ambient co-culture temperatures, and survive but not replicate at 4°C, similarly to Y. pestis-A. castellanii coculture assays (Fig 2). This will also be similar to the ability of L. pneumophila (39, 40) and other bacterial pathogens (4, 41, 42) to survive in environmental FLA. If the burrow Acanthamoeba is unable to support survival and replication of Y. pestis, we will test the burrow Hartmanella FLA species. Technical alternatives: Flow cytometry could be used to assess survival and

replication of Y. pestis in trophozoites as has been achieved in other studies defining interactions

between bacterial pathogens and FLA (4, 27). Overall we expect that this aim will provide biological evidence that *Y*. *pestis* can colonize environmentally relevant amoeba species.

**Specific Aim 2** Determine if the ability of Y. pestis to survive long term and/or replicate in amoeba can be recapitulated in a simulated burrow soil environment.

**<u>Rationale</u>**: During sterile and highly controlled laboratory culture *Y. pestis* is able to survive and replicate in amoeba. However it is not known if a natural soil environment can support prolonged intra-amoebal survival of *Y. pestis*.



<u>Fig. 4.</u> GFP-*Y. pestis* within *A. castellanii* seeded on agar plates. Image B is a magnification of the red block in image C.

Experimental design: The soil collected from rodent burrows (described in preliminary studies) will be used. Temperature (°C) and relative humidity (%RH) will be maintained within the range observed in rodent burrows during epizootic (~25°C; 68%RH) and guiescent plague cycles (4-10°C, 80%RH). To allow fluorescent microscopic detection of Y. pestis a green fluorescent protein tagged Y. pestis (GFP-Y. pestis; shown in Figs 3 and 4) will be grown and seeded in mouse blood at a concentration of that present in a bacteremic mouse (108-109 cfu/mL). One mL of infected blood will be added to 1g soil. Amoeba will be added to one replicate of soil at ~10<sup>11</sup> trophozoites/g soil to produce a multiplicity of infection of 100, similar to our axenic coculture experiments (Fig 2); the other replicate will serve as a control for bacterial survival in soil lacking FLA. Similarly a soil sample, containing amoeba only, will serve as a control for changes in amoeba numbers in the absence of bacteria. Over a period of two months in which the experimental soil environment will first be incubated at epizootic conditions for two weeks, followed by inter-epizootic conditions for 1 month and then epizootic conditions again for two weeks, soil samples will be collected and processed every 3-4 days to detect changes in bacteria and amoeba numbers as follows: (i) total genomic DNA will be isolated using Mobio Powersoil PowerLyzer® Kits and quantitative real-time PCR of unique target genes encoded on their chromosomes will be undertaken to determine changes in copy numbers of bacteria and amoeba; (iii) as a qualitative determination and for detection of viable bacteria, wetted soil samples will be added to agar wells and Y. pestis infected amoeba allowed to proliferate. Fluorescent microscopic analysis of the agar plate has the ability to identify if GFP-Y. pestis are present within amoeba. Burrow amoeba was originally isolated from soil using agar plates. Our test experiment using A. castellanii and GFP-Y.pestis grown axenically, and then seeded on agar plates proves feasibility of identifying intra-amoebal fluorescing Y. pestis on agar plates. (Fig 4). This method can be advantageous in detecting low numbers of infected amoeba as these can subsequently be amplified in culture and processed as in Aim 1. Experiments will initially be optimized using A. castellanii and tested using burrow amoeba. Analysis and alternatives: We expect to identify increases or no alteration in bacterial numbers under epizootic conditions ideal for amoeba proliferation. Under inter-epizootic conditions it's expected that there will be decreases or no alteration in bacterial numbers as conditions should be less favorable for proliferation. However, our hypothesis that amoeba serve as a Y. pestis reservoir will still be accepted as long as viable intra-amoebal bacteria are demonstrated at the end of 3 months, irrespective of whether bacterial numbers are altered in either condition.

**Overall outcomes:** These experiments will provide the basis for specific targeting of amoeba in rodent burrows and for quantitatively defining levels of intra-amoebal bacteria expected in burrows during plague cycles. This is of paramount importance for detecting the *Y. pestis*-amoeba interaction in prairie plague foci in order to predict plague re-emergence in such areas. The ability to predict a plague epizootic could inform which prairie areas are viable for recovery of black-footed ferrets, or implementation of early interventions that prevent disease spread and extinction of prairie dog colonies. This will have the overall impact of reducing risk of disease spread to other susceptible wildlife species e.g. mountain lions or to domestic animals e.g. cats and dogs.

5. **Timeline:** During Year One, Aim 1 will be performed and accomplished. Concurrently, optimization of experiments in Aim 2 will be undertaken. In Year Two final investigation into the dynamics of bacterial survival in amoeba in a burrow soil simulating environment and manuscript preparation and submission will be accomplished

## **Morris Animal Foundation**

## **Animal Involvement Justification**

(From the proposal guidelines, single-spaced, no page limit)

Morris Animal Foundation (MAF) is dedicated to funding scientifically sound, relevant and humane studies that specifically address the health and well-being of animals. All studies receiving funding must follow MAF's Health Study Policy for Animals Involved in Research (adopted October 18, 2008), which was written to ensure that each and every animal involved in a MAF funded health study receives excellent, compassionate care throughout the study. MAF shall not fund health studies which require euthanasia as an endpoint or the induction of disease or injury, unless the nature of the disease or condition to be studied is of such significance for improving animal health that such means are justified, and that meaningful information can be obtained in no other way. Furthermore, MAF will not fund any study that induces or allows pain or distress unless such pain or distress can be controlled by appropriate anesthetic, analgesic, tranquilizing drugs, or nursing care. <u>Click here</u> for the full Health Study Policy.

- A. If this study does not involve live animals please indicate here by N/A: N/A
- **B.** Does this study involve biological samples, tissues, etc.? N/A, Mouse blood will be acquired from commercial vendor (Bioreclamation, New York)

**If yes**, describe in detail what samples will be used and where & how they will be (or were) acquired. Note: Morris Animal Foundation reserves the right to request a copy of the Institutional Animal Care and Use Committee (IACUC) application/approval and other relevant applications/approvals (e.g., wildlife permit) covering the original collection of samples, including archived samples. MAF reserves the right to request IACUC (or equivalent) review and approval for any Foundation study *regardless of the Institution's requirements*. This would include the use of archived samples as well as clinical trials.

- C. If this study involves live animals, succinctly address the following: (please restate the questions and directives).
  - 1. What species will be studied?
  - 2. State the status of your IACUC application/approval. All recipients of MAF funding will be required to submit the entire IACUC protocol and document. A copy of the IACUC approval should not be included with the application, but it is required before funding can be awarded.
  - 3. List the USDA category for pain and distress (B, C, D, E): \_\_\_\_\_

Note: Any study beyond category C will require review by MAF's Animal Welfare Advisory Board (AWAB). In general MAF does not fund studies beyond category C (category D studies will only be considered if they conform with MAF's Health Study Policy, category E studies will not be considered).

4. Does this proposal involve client-owned animals?

If yes, the protocol for client-owned animals must be approved by the appropriate peer review committee before the project is funded. *If this proposal involves client-owned animals, an informed client consent form must be submitted with this proposal. For a suggested list of items to be considered in an informed client consent form, click here.* 

- 5. Explain how animals will be acquired (e.g., client-owned, USDA licensed breeder, institutional "herds" or "colonies") and verify that the animals are suitable for the study (e.g., have no physiologic, physical or pharmacologic issues that would interfere with results)
- 6. How many animals will be used?
  - a. Summarize numerical justification
- 7. Does this study induce disease, injury, pain or distress in animals? Note: any study requiring the induction of disease, injury, pain, or distress will have an additional evaluation by MAF's AWAB.

If yes,

- a. Defend the necessity of experimental design
- b. Explain how pain and/or distress will be controlled
- c. Justify that no alternative, including clinical studies, can be used to accomplish study objectives and the disease/condition to be studied is of such significance for improving the health of the species.
- 8. Explain the environment and housing conditions (quality of life) in which the animals will live (address species-appropriate exercise, enrichment, socialization, veterinary care, etc.)
- 9. What will happen to the animals upon completion of the study?

If adoption, explain the adoption process. Provide assurance that whenever possible and when in the animal's best interest, investigators shall make companion animals available for adoption at the end of the study or return the animals to the owner/responsible agency in an environment that promotes animal welfare and excellent quality of life.

- 10. If euthanasia, provide the following additional information (note: any study requiring euthanasia as an endpoint will have an additional evaluation by a MAF's AWAB.
  - i. Total number that will be euthanized and justification for numbers
  - ii. Method of euthanasia
  - iii. Justification that no alternatives can be used to accomplish study goal(s) and that the disease/condition to be studied is of such significance for improving the health of the species that a terminal endpoint is deemed necessary.
  - iv. Reason for euthanasia in lay language (this wording may be shared with staff, donors and media)
  - v. Provide objective criteria for determining when euthanasia is appropriate or necessary (note: Morris Animal Foundation wants assurance that an animal will not be allowed to suffer and that monitoring for pain and suffering is adequate)

Note: Morris Animal Foundation does not consider the use of CO2 alone to be an appropriate method of euthanasia

#### Please note:

- 1. If an animal is used in an invasive study, MAF may require that a guarantee is provided, through principal investigator and institutional signatures that the animal will not participate in any future invasive study or procedure
- 2. MAF does not allow inclusion of ancillary data in MAF funded research that includes animal use protocols not in agreement with our Health Study Policy, even if it is obtained using other funding sources.
- 3. Morris Animal Foundation considers euthanasia acceptable when an animal develops unanticipated illness or injury that results in pain and suffering that cannot be alleviated with standard veterinary interventions.

**VI. Recombinant DNA/Biohazards**: Recombinant DNA will not be used in this study. The *Y. pestis* strains used in this study are designated avirulent and will be used at Biosafety Level 2 (BSL2). The BSL2 practices using avirulent *Y. pestis* have been approved by the Washington State University Institutional Biosafety committees

## VII. Facilities and equipment:

## Paul G. Allen School for Global Animal Health, College of Veterinary Medicine, WSU

The Paul G. Allen Center for Global Animal Health, opened in 2012, is a 62,000-square-foot state-of-theart infectious disease research facility designed and equipped to meet today's standards for investigating pathogens and emerging diseases. The facility includes administrative offices and a CDC and USDA certified Biosafety Level 3 (BSL3) laboratory on the first floor, and two floors of BSL2 laboratories, meeting rooms, office space, and open areas to encourage direct interactions among the students, faculty and staff. In addition to individual faculty laboratories, there are common equipment rooms and shared facilities including licensed work sites for bioinformatics.

**Laboratory facilities:** Dr. Vadyvaloo has an assigned laboratory in the Allen Center. Additional resources and laboratories are available to Dr. Vadyvaloo throughout the College of Veterinary Medicine (CVM) complex. The Allen School and other CVM laboratories are fully equipped for contemporary research in molecular biology, infectious diseases, and immunology. The BSL2 laboratories have been approved by the Institutional Biosafety Committee for pathogen use, recombinant DNA experiments and radioisotope use.

**Equipment:** The allocated laboratory space is equipped with four 4-ft biological safety cabinets, four CO<sub>2</sub> incubators, various bacterial incubators and shakers for recombinant work in *Escherichia coli* and *Yersinia pestis*, a Thermolyne Locator Jr Plus nitrogen freezer for cryostorage of amoeba cell lines, Thermo Revco -86°C freezers for long term storage of strains and samples, standard DNA, RNA and protein electrophoresis equipment, 2 BioRad C1000Touch PCR cyclers and a CFX96/384 Real Time PCR System, various Eppendorf centrifuges, biospectrophotometer, Agilent Bioanalyzer (RNA/DNA quality analysis) and a Biorad GenePulser Xcell for electroporation, Nanodrop (DNA/RNA quantification), pipettes (single and multi-channel), EVOS fluoresence microscope, spectrophotometer, and heating blocks. Gamma counters, HPLC and FPLC are available nearby.

Additional equipment is available for use in shared facilities in the Allen Center including a Leica DM4000 epifluorescence upright microscope with 4 laser lines: (1) Excitation 360/40, emission 470/40 (for DAPI and Hoechst); (2) Excitation 480/40, emission 527/30 (for Alexa 488, FITC, GFP); (3) Excitation 545/40, emission 610/75 (for Alexa 546, Alexa 568, mCherry, DsRed); (4) Excitation 640/30, emission 690/50 (for Cy5, Alexa 647). Also available are TECAN Infinite M1000 fluorescence/luminescence microplate reader, a Millipore guava easyCyte<sup>™</sup> HT flow cytometer, a BioRad ChemiDoc<sup>™</sup> MP gel imaging system, Sorvall WX Ultra 100 and MTX 150 ultracentrifuges, Sorvall high speed centrifuges, an Illumina MiSeq personal sequencer, Zeiss Primovert microscope for tissue culture, and Lonza 4D-Nucleofector.

**Office:** The PI has a private 150 sq. ft. office space located next to her research laboratory. Shared office space is also available for technicians, postdocs, and graduate students working in the laboratory

**Computer:** Dr. Vadyvaloo has networked computers in her laboratory and office including multiple specialized software packages, and wireless connectivity is available throughout the building.

#### Additional resources available on the WSU campus

A Leica TCS SP8 X point scanning confocal microscope with 7 laser lines is available in nearby BSL2 lab space in the CVM *Department of Integrative Physiology and Neuroscience*.

*Microscopy and Imaging*: The Franchesci Center maintains the full complement of microscopy services and instrumentation. This includes confocal microscopes (BioRad MRC 1024; Zeiss LSM 510), 2 transmission electron microscopes (FEI TEM T20, Philips CM-200), 2 scanning electron microscopes (Hitachi S-570, FEI Quanta 220F) electron microscopes, Fluorescence microscope (Leica) and laser dissection microscope (PALM MicroBeam Laser Dissection Core). All are fitted with imaging instrumentation. The full-set of tissue processing for these microscopy needs are available at the Center. All necessary ancillary equipment, computers for image processing and analysis are maintained for student and faculty use. The center provides project consultation and has a skilled staff to assist students and faculty in a wide range of research projects

Laboratories in the neighboring *Animal Diseases and Biotechnology Facility* are fully equipped an approved for research in molecular biology, infectious diseases, and radioisotope use, as described above.

### VIII. REFERENCES

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9. **Eisen RJ, Gage KL.** 2009. Adaptive strategies of *Yersinia pestis* to persist during inter-epizootic and epizootic periods. Vet Res **40**:1.

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infection of the amoebae Acanthamoeba castellanii, Hartmannella vermiformis, and Naegleria lovaniensis. Infect Immun 81:1399-1410.

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## IX. PROPOSAL BUDGET

Category		Year 1	Year 2	Total	
<b>Perso</b> 1.	<u>nnel:</u> Principal investigator (Viveka Vadyvaloo, PhD)*				
2.	Co-investigator #1 (Javier Benavides- Montano, DVM)				
3.	Technician Salary (40%) Fringe benefits (33.2%)				
Tot	al Salaries & Wages				
<u>Suppl</u> 1.	ies & Expenses: Consumables (plastic ware, tips, tubes, tissue culture flasks, etc.)				
2.	Chemicals and media components (glucose, magnesium chloride, brain- heart infusion broth, sodium chloride, etc.)				
3.	Molecular reagents (Mobio Powersoil Kit, qPCR reagents, etc.)				
4.	Publication/Page charges				
5.	Annual Usage Fee for the Microscopy facility				
6.	Microscopy reagents				
Provide justification in the designated section. Total Supplies & Expenses:					
Anima Anir Anir Tota	al Use & Care: mal Purchase: mal Per diem: al Animal Care:				
Subtotal of All Categories:					
Maximum of 8% - Indirect Costs:**					
Grand Total Requested from MAF:					

## X. Itemized Budget Justification

#### Salaries and benefits:

#### Principal Investigator (Viveka Vadyvaloo, PhD)

Dr. Vadyvaloo has >9 years' experience working on *Y. pestis*. As PI, she will coordinate, supervise and participate in generating experimental data for the proposed work. She will oversee and advise all technical aspects of the research outlined in the specific aims. Dr. Vadyvaloo will be responsible for data analysis and interpretation, as well as writing and submitting reports and manuscripts for publication. Dr. No salary or benefits are

requested.

#### Co-Investigator (Javier Benavides-Montaño, DVM)

Mr. Benavides-Montanõ has 2 years' experience with *Y. pestis* in amoeba and is responsible for generating the preliminary data presented in this proposal. Under the supervision of Dr. Vadyvaloo, he will assist in conducting the experiments described in both aims, will participate in the data analysis and interpretation, and manuscript preparation. No salary or benefits are requested.

#### Laboratory technician (To be determined)

Under the direct supervision of Dr. Vadyvaloo the technician will assist in conducting the experiments outlined in Aims 1 and 2, and will provide support to Mr. Benavides-Montaño on all experimental aspects of the project. The technician will also maintain amoeba and bacterial culture stocks, prepare media, and oversee general laboratory BSL-2 protocol and maintenance. Salary **Example** and **Example** benefits are requested to cover the cost of the technician at 40% effort, for a total cost of **Example** 

#### Supplies and expenses:

A total is requested to cover the supplies and expenses associated with the completion of the specific aims in this proposal.

- 1. <u>Consumables and plastic-ware:</u> tissue culture flasks, tubes, pipets, pipet tips, petri plates etc.
- 2. <u>Chemicals and reagents:</u> chemicals to make media (NaCl, MgCl, glucose, vitamins, minerals etc.), brain heart infusion, etc.
- 3. <u>Molecular biology reagents:</u> Mobio Powerlyzer® Powersoil® soil DNA extraction kit, primers and probes for qRT-PCR, qRT-PCR reagents, DNA stain, DNA loading buffer. **The second seco**
- 4. <u>Publication and page charges:</u> is requested in year 2 to defray publication expenses.
- 5. <u>Use of the microscopy (electron) facility:</u> per year is requested to pay the annual usage fee of the Franceschi Microscopy & Imaging Center.
- 6. <u>Microscopy reagents:</u> (uranyl acetate, electron grids, cacodylate buffer, etc.) is requested to cover the cost of microscopy reagents over the two year period.

#### **XI. Other Support**

#### Current support:

Project number:

Source: College of Veterinary Medicine Washington State University Intramural Grant program

Overlap: The above funded project has conceptual and scientific overlap with the currently proposed study. In terms of % effort and resources there is no overlap because the latter is focused on understanding survival and replication of *Y. pestis* in ecologically relevant amoeba that have been identified by studies that have been accomplished from the currently funded project.

Pending support:		
Project number:		

Overlap: No overlap exists between the above study and the currently proposed study.

## XII. Prior MAF Support during the last three years

None

## **BIOGRAPHICAL DATA**

#### NAME: Viveka Vadyvaloo

#### POSITION/ROLE ON PROJECT: Principal Investigator

#### CURRENT POSITION: Assistant Professor, Paul G. Allen School for Global Animal Health Washington State University, PO Box 647090, Pullman, WA 99164-7090

#### EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
University of Durban-Westville, Durban, South Africa	BS	12/1995	Microbiology, Human Physiology
University of Kwazulu-Natal, Pietermaritzburg, South Africa	MS	12/1999	Microbial genetics
University of Stellenbosch, Stellenbosch, South Africa	PhD	06/2003	Biochemistry
Howard Hughes Medical Institute at UCLA, Los Angeles, California	Postdoctoral Fellow	08/2004	Biochemistry
NIH/NIAID/ Rocky Mountain Laboratories, Hamilton, Montana	Postdoctoral Fellow	09/2005	Bacterial pathogenesis
NIH/NIAID/Rocky Mountain Laboratories, Hamilton, Montana	Postdoctoral Fellow	09/2008	Vector-borne disease; plague

#### **Positions and Honors**

#### Positions and Employment

- 2009 2010 Research Assistant Professor, Paul G. Allen School for Global Animal Health, Washington State University, Pullman, WA
- 2010 present Assistant Professor, Paul G. Allen School for Global Animal Health, Washington State University, Pullman, WA

#### Other Experience and Professional Memberships

- 2003 Member, American Society of Microbiology
- 2010 Member, North West Regional Center of Excellence
- 2011 Member, Entomological Society of America

#### <u>Honors</u>

- 1997AECI MS Fellowship, (AECI/CSIR South Africa)
- 2000 Prestigious Equity PhD Fellowship, National Research Foundation of South Africa
- 2014 NSF ADVANCE at WSU External Mentor Program Award, NSF/WSU
- 2014 International Travel Award, Washington State University

## **Selected Peer-Reviewed Publications**

- Martínez-Chavarría LC, Vadyvaloo V. 2015. Yersinia pestis and Yersinia pseudotuberculosis infection: a regulatory RNA perspective. Front Microbiol. 6:956. doi: 10.3389/fmicb.2015.00956. eCollection 2015. Review. PubMed PMID: 26441890; PubMed Central PMCID: PMC4585118.
- Vadyvaloo V, Hinz AK. 2015. A LysR-Type Transcriptional Regulator, RovM, Senses Nutritional Cues Suggesting that It Is Involved in Metabolic Adaptation of *Yersinia pestis* to the Flea Gut. PLoS One. 10(9):e0137508. doi: 10.1371/journal.pone.0137508. eCollection 2015. PubMed PMID: 26348850; PubMed Central PMCID: PMC4562620.
- Santos-Montañez J, Benavides-Montaño JA, Hinz AK, **Vadyvaloo V**. 2015. Yersinia pseudotuberculosis IP32953 survives and replicates in trophozoites and persists in cysts of Acanthamoeba castellanii. FEMS Microbiol Lett. 362(13):fnv091. doi: 10.1093/femsle/fnv091. Epub 2015 May 29. PubMed PMID: 26025069.
- Vadyvaloo V, Viall AK, Jarrett CO, Hinz AK, Sturdevant DE, Joseph Hinnebusch B. 2015. Role of the PhoP-PhoQ gene regulatory system in adaptation of *Yersinia pestis* to environmental stress in the flea digestive tract. Microbiology. 161(6):1198-210. doi: 10.1099/mic.0.000082. Epub 2015 Mar 24. PubMed PMID: 25804213.
- Bobrov AG, Kirillina O, Vadyvaloo V, Koestler BJ, Hinz AK, Mack D, Waters CM, Perry RD. 2015. The Yersinia pestis HmsCDE regulatory system is essential for blockage of the oriental rat flea (Xenopsylla cheopis), a classic plague vector. Environ Microbiol. 17(4):947-59. doi: 10.1111/1462-2920.12419. Epub 2014 Mar 11. PubMed PMID: 25586342; PubMed Central PMCID: PMC4295937.
- Martínez LC, Vadyvaloo V. 2014. Mechanisms of post-transcriptional gene regulation in bacterial biofilms. Front Cell Infect Microbiol. 4:38. doi: 10.3389/fcimb.2014.00038. eCollection 2014. Review. PubMed PMID: 24724055; PubMed Central PMCID: PMC3971182.
- Rempe KA, Hinz AK, Vadyvaloo V. 2012. Hfq regulates biofilm gut blockage that facilitates flea-borne transmission of Yersinia pestis. J Bacteriol. 194(8):2036-40. doi: 10.1128/JB.06568-11. Epub 2012 Feb 10. PubMed PMID: 22328669; PubMed Central PMCID: PMC3318476.
- Vadyvaloo V, Jarrett C, Sturdevant DE, Sebbane F, Hinnebusch BJ. 2010. Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. PLoS Pathog. 6(2):e1000783. doi: 10.1371/journal.ppat.1000783. PubMed PMID: 20195507; PubMed Central PMCID: PMC2829055.
- Vadyvaloo V, Jarrett C, Sturdevant D, Sebbane F, Hinnebusch BJ. 2007. Analysis of Yersinia pestis gene expression in the flea vector. Adv Exp Med Biol.;603:192-200. Review. PubMed PMID: 17966415.
- Erickson DL, Waterfield NR, Vadyvaloo V, Long D, Fischer ER, Ffrench-Constant R, Hinnebusch BJ. 2007. Acute oral toxicity of *Yersinia pseudotuberculosis* to fleas: implications for the evolution of vector-borne transmission of plague. Cell Microbiol. 9(11):2658-66. Epub 2007 Jun 24. PubMed PMID: 17587333.

## **BIOGRAPHICAL DATA**

#### NAME: Javier Antonio Benavides Montaño

#### POSITION/ROLE ON PROJECT: Co-Investigator

#### CURRENT POSITION: PhD Student, Paul G. Allen School for Global Animal Health Washington State University, PO Box 647090, Pullman, WA 99164-7090

#### EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	Year of Graduation	FIELD OF STUDY
Universidad de Caldas, Manizales, Colombia	BS	1999	Veterinary Medicine and Animal Production Science
University of Manizales, Caldas, Colombia	M.Ed	2005	Education and University Teaching
Federal University of Viçosa – Brazil	MSc	2006	Veterinary Medicine

## **Positions and Honors**

## Positions and Employment

2000-2004 Diego Villegas Toro. Clinical hospital, Universidad de Caldas. Residency in small animal medicine; Clinical Parasitology; Teaching Assistant.

- 2007-present Award Academy Excellence I 07, Faculty of Agriculture Science (Palmira), National University of Colombia.
- 2011-present Fulbright Scholar, Washington State University, Pullman, WA, USA

#### <u>Honors</u>

2010 Best Research Award. XXXVII Congreso Sociedad Colombiana de Entomologia (SOCOLEN), Bogota, July 2010.

## Selected Peer-Reviewed Publications (English language)

- Santos-Montañez J, **Benavides-Montaño JA**, Hinz AK, Vadyvaloo V. 2015. Yersinia pseudotuberculosis IP32953 survives and replicates in trophozoites and persists in cysts of Acanthamoeba castellanii. FEMS Microbiol Lett. 362(13):fnv091. doi: 10.1093/femsle/fnv091. PubMed PMID: 26025069.
- Lu X, Liu Q, Benavides-Montaño JA, Nicola AV, Aston DE, Rasco BA, Aguilar HC. 2013. Detection of receptor-induced glycoprotein conformational changes on enveloped virions by using confocal micro-Raman spectroscopy. Journal of Virology. 87(6):3130 – 3142. doi: 10.1128/JVI.03220-12. PMCID: PMC3592171
- Liu Q, Stone JA, Bradel-Tretheway B, Dabundo J, **Benavides-Montaño JA**, Santos-Montañez J, Biering SB, Nicola AV, Iorio RM, Lu X, Aguilar HC. 2013. Unraveling a three-step spatiotemporal mechanism of triggering of receptor-induced Nipah virus fusion and cell entry. Plos Pathogens. 9(11):e1003770. Doi: 101371/journal.ppat.1003770. PMCID: PMC3837712

- Benavides-Montaño JA, Rocha HGR, Campos VEB, Ferreira APBR, Souza e Silva CH, Carvalho GD, et al. 2009. Humoral immune responses to the synthetic immunogens SBbo23290 (*Babesia bovis*) and SBm7462 (*Rhipicephalus* (*Boophilus*) *microplus*). Veterinary Immunology and Immunopathology. 128(1-3):284-285. doi:10.1016/j.vetimm.2008.10.160
- Benavides-Montaño JA, Rocha HGR, Nogueira da Gama M, Campos VEB, Ferreira APBR, Carvalho GD, et al. 2009. Biological parameters of ticks collected from animals immunized with peptides SBbo23290 and SBm7462. Veterinary Immunology and Immunopathology. 128(1-3):285-286. doi:10.1016/j.vetimm.2008.10.161

# Fellowship Training

#### I. Title and Abstract:

Title: Integrated metabolomic and genomic approach to metabolic variation across horse breeds

**Rationale:** Equine Metabolic Syndrome (EMS) is defined by a clustering of clinical signs, namely hyperinsulinemia, insulin resistance and adiposity, which predispose horses to the development of laminitis. Certain breeds appear to be more susceptible to EMS, while other breeds seem to be at lower risk. Genetic selection in horses has resulted in metabolic and athletic phenotypes that allowed horses to efficiently perform different types of work. These underlying metabolically efficient, or "thrifty" alleles, in particular those that regulate energy intake, storage and use, coupled with changes in equine husbandry practices in recent times, including dense high caloric feed and limited exercise, may explain much of the increasing prevalence of equine metabolic disease phenotypes in modern environments.

**Hypothesis/Objectives:** We *hypothesize* that breed differences in key metabolic phenotypes are due to high frequencies of alleles that modify metabolic traits. Our *objectives* are to 1) further dissect the metabolic differences between breeds at the molecular level using total serum metabolite profiling; and 2) use these breed-specific molecular metabolic profiles to identify candidate genes underlying breed metabolic differences.

**Study Design:** In <u>**Objective 1**</u> serum metabolite profiles will be analyzed before and after an oral sugar test in 274 horses from 5 breeds with distinct metabolic phenotypes. These data will be used to 1) identify the metabolites and metabolic pathways that are significantly different between breeds using functional annotation and mapping of metabolites to known pathways, pathway and metabolite set enrichment analysis, network analysis, and pathway activity profiling; and 2) to correlate the metabolite/pathway differences with the previously identified breed differences in key hormonal and biochemical measurements. In <u>**Objective 2**</u> candidate genes responsible for metabolic differences between breeds will be identified by using high-density SNP genotype data to locate genomic regions and specific haplotypes that are highly differentiated between breeds. Metabolites and metabolic pathways that are significantly different between breeds will then be used to provide context for narrowing the focus to specific candidate genes within these genomic regions.

**Preliminary Data:** Our data demonstrate significant differences among Morgans, Arabians, Welsh ponies, Tennessee Walking Horses and Quarter Horses in EMS-defining metabolic traits (e.g. insulin dynamics, lipid metabolism, adipokines) that mirror EMS risk. We have also demonstrated the use of SNP genotype data to identify regions of breed differentiation/selection across a wide breed panel, and across Quarter Horses and Welsh Ponies, and provided examples in the *GYS1* and *MSTN* genes that selection for certain performance traits results in near-fixation of alleles that alter energy metabolism. Finally, we have demonstrated the potential for serum metabolomic data to lead to insight into the metabolic differences between horses and clinical phenotypes.

**Expected Results:** We expect to detect major breed differences in metabolites/metabolic pathways that are correlated to previously identified biochemical and hormonal differences. We also expect to identify and prioritize candidate genes within genomic regions of interest that influence a spectrum of metabolic traits, particularly the susceptibility to metabolic syndrome.

**Budget and Timeline:** objective.

Approximately one year is required to achieve each

**Potential Impact for Animal Health:** Elucidating the evolution of the genetic basis of metabolic efficiency and metabolic syndrome is a novel, unexploited approach to the study of the genetic basis of obesity, energy dysregulation and EMS. This project will provide novel insights into disease biology, allowing the identification of new therapeutic targets, and increasing our understanding of the pathophysiology of EMS and its associated clinical features. Moreover, the identification of genes underlying the EMS phenotype will also directly impact equine health by allowing for the development of genetic tests to identify horses at risk for the development of obesity and laminitis prior to the onset of clinical disease.