COOPERATIVE BIOLOGICAL RESEARCH
Project Proposal
GG-19 – Form A/Science Plan

Revised Version
Re-submitted to DTRA by CH2MHILL on 01 June 2012

Project Title

Epidemiology and Ecology of Tularemia in Georgia

Project Summary

The overall scientific goals of this study are to study the epidemiology of human and animal tularemia in Georgia. We will accomplish this through several specific objectives. First, we will examine the seroprevalence of tularemia among exposed (people living in geographic areas known to be natural foci for Tularemia) and healthy individuals; we will estimate risk factors for seropositivity. In parallel, we will also establish active surveillance for human tularemia clinical cases with the goal of increasing the efficiency and diagnostic capability to identify the disease; we will collect isolates from human cases for comparison to both current environmental isolates and historical isolates (currently in the NCDC collection). From an environmental prevalence emphasis, we will establish active surveillance for *F. tularensis* in the environment including small rodents and associated vectors and to identify the sources of outbreaks among humans. This effort includes linking environmental and animal cases with human cases. Additionally, in all strains isolated we will evaluate diagnostics methods for detecting *F. tularensis*, and, monitor patterns of antimicrobial resistance with these isolates. There will be a bacteriophage component to this project that will study isolated *Francisella tularensis* strains strain by genomic sequencing, proteomics analysis and phage discovery (expanding our efforts from our discovery of lytic Francisella bacteriophage). The result of this project will be a more accurate assessment of the base-line prevalence and geographic distribution of *F. tularensis* pathogens in humans and animals; and an improved recognition and detection of the disease in this region. Both goals will be achieved by providing better laboratory diagnostics, enhanced GIS data, and modern laboratory-based characterizations for *F. tularensis*.

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Detailed Project Information

Project Description (Introduction and Overview)

Background

Tularemia is a zoonotic infection caused by *Francisella tularensis* - a small, faintly staining, Gram-negative, non-motile, non-spore-forming, pleomorphic bacterium, typically appearing as short rods or coccoid forms[1]. *F. tularensis* is an aerobic bacterium and considered as a fastidious organism due to its requirement of Cysteine for enhanced growth. The geographical distribution of *F. tularensis* spans the entire Northern Hemisphere. At present, there are four recognized subspecies of *F. tularensis: tularensis, holarctica, mediaasiatica*, and *novicida*. Each subspecies is predominantly associated with a particular geographical distribution. *F. tularensis* subsp. *tularensis* is predominately found in North America, yet was recently recovered in central Europe. *F. tularensis* subsp. *holarctica* is found over much of the Northern Hemisphere, while *F. tularensis* subsp. *mediaasiatica* has only been isolated in Central Asian republics. *F. tularensis* subsp. *novicida* is rarely isolated and prior to 2003 appeared restricted to North America. *F. tularensis* is included among the top six agents showing potential for great adverse public health impact if used as a bioterrorism agent.

Georgia offers a unique opportunity to study the epidemiology of the *holarctica* subspecies as tularemia infections arising from this variant (type B) have been long established, further, there have been detailed investigations of widespread endemic foci in south and eastern Georgia. It is here that the current risk and potential for preventive measures is in need of assessment following the cessation of local vaccination and rodent control programs. Humans are accidental hosts; infection occurs following contact with infected animals or invertebrate vectors. After incubation period of 2-10 days, patients usually develop fever, headache, malaise, and can be accompanied by ulcerative or popular skin lesion, lymphadenopathy, hepatosplenomegaly, pharyngitis, and etc[2]. Depending on the portal of entry of *Francisella* infection, six different clinical syndromes are identified[3]: Glandular tularemia, Ulceroglandular tularemia, Oropharyngeal tularemia, Oculoglandular tularemia, Pneumonia and influenza tularemia, and, Typhoid tularemia. Post-infectious syndromes with persistent neuropsychiatric disturbances and chronic fatigue syndrome have been described in patients who were untreated or received therapy during late phase of their disease.[4, 5, and 6]

Epidemiology of Tularemia

Tularemia outbreaks and epizootics occur sporadically in scattered but widespread regions in the Northern Hemisphere. While the geographic distribution of *F. tularensis* foci is reasonably documented, some local aspects of the epidemiology of tularemia remain unclear due to the difficulty in fully understanding the potential diversity of local reservoirs and modes of transmission of *F. tularensis* (Table 1). While there may be difficulty in knowing the risk factors that exist to identify individuals who may be infected, there is an extensive literature on the risk factors, vectors and reservoirs for the four epidemic types (I - IV) of tularemia, the common risk factors relate to outdoor activity in endemic areas, usually from tick and mosquito bites, handling infected hares or rodents, drinking contaminated water and aerosol exposure from moving rodent contaminated hay and laboratory accidents.

Table 1: Epidemic Characteristics of Tularemia

Origin of epidemic	Groups of the population exposed to the infection	Mode of infection	Predominant form of the disease and location of the buboes	Time of origin of the outbreak	Source of infection	The principle methods of prophylaxis and eradication of the epidemic
Type I Epidemic resulting from wounds of mucous membrane infection – contact epidemic						
The water vole fur trade. Trapping of hares and other animals susceptible to tularaemia in food-plain foci	Persons engaged in hunting and handling the carcasses	Contact with the carcass or dead body of an infected animal	The bubonic form. Buboes localized mainly in the axillary and cubital regional nodes	At the time of the spring floods and the hunting season	Water voles, hares and other animals	Prophylactic vaccination of vulnerable groups
Rodent control work or apizootic disease surveys	Persons engaged in work of this type	As above	As above	The time of year at which the work is carried out	Murine rodents	Attention to personal prophylaxis
Hay-cutting in marshy meadows	Persons engaged in this work. The incidence is normally low.	Contact with contaminated water and other substrates	The bubonic form with buboes mainly in the inguinal region	Spring and summer (i.e. the hay cutting season)	Murine rodents	
Type II Epidemic in which the infection is transmitted by an arthropod vector (Vector-borne)						
The presence of human beings in the characteristic biotopes of arthropod vectors	Random members of groups working near water, marshy ground, or bushes and trees	The bit of mosquitoes, horse flies, and other arthropod vectors	The ulcerous bubonic form. The ulcers are located mainly on exposed parts of the body if dipteran insects are responsible for the bites	From July to September. The period when arthropods are most active	The water vole and murine rodents	Protection from bites by the use of repellents and other means; prophylactic vaccination of the inhabitants of natural foci of tularaemia
Type III Epidemic in which infection is contracted orally (water and food-borne epidemic)						
Contamination of water by the discharges or corpses of disease rodents	The disease is restricted to people using water from a contaminated source. Usually a large proportion of those drawing water from one particular source contract the disease within a short space of time.	The use of contaminated water in its raw state for drinking purposes	The anginous-bubonic and the intestinal forms. An intermediate form is probably the most common, however	Summer and autumn usually. Winter outbreaks have also been recorded	Murine rodents and more rarely water voles	Sterilization of water and purification and disinfection of wells. The chlorination of piped water supplies, rodent control and prophylactic vaccination of the inhabitants of natural foci
Contamination of foodstuffs by the discharges of diseased rodents	People consuming contaminated food stuffs which have not been adequately cooked. In contract to water-borne epidemics the incidence is low	Consumption of contaminated foodstuffs	The intestinal form is the most common but the anginous-bubonic form may also be observed	Autumn and winter	Murine rodents	Protections of foodstuffs from the activities of rodents, extermination of rodents, and prophylactic vaccination of the inhabitants of natural foci
Type IV Epidemic where infection is the result of inhalation of contaminated air (aspiration epidemic)						
The threshing of stacked corn, the carting of straw and the handling of threshed grain and rodent-contaminated vegetables	The disease is confined to persons engaged in this type of work or sleeping on contaminated straw. A high incidence of disease is normally associated with epidemics of this type	Inhalation of droplets or dust contaminated by the discharges of diseased rodents	Tularaemia of the respiratory tracts and occasionally of the intestinal canal	Winter (early) spring and sometimes autumn)	Murine rodents	The use of respirators during work, the use of fir branches instead of straw for bedding, priophylactic vaccination of the inhabitants of natural foci of tularaemia and the extermination of rodents

F. tularensis transmission to man occurs in residents and travelers to these endemic areas in Georgia, where it would be expected to find cases following the bites of ticks, mosquitoes and rodents or from handling infected hares or skinning carcasses. Water-borne outbreaks have occurred in Georgia and elsewhere from drinking water from contaminated streams or wells especially at times of civil disruption and damage to supply systems and wells. Individual cases of pulmonary tularemia are documented in Georgia because of airborne infection after moving

rodent-contaminated hay and when thrashing corn. Typhoid tularemia has been diagnosed during at least two waterborne outbreaks in Georgia. Additionally, the epidemiology of the disease can appear to have changed over time, necessitating continued study of the endemic foci in any particular country. This is due to two factors: under ascertainment of cases both through lack of clinical knowledge and access to diagnostic test facilities, and the fact that the clinical epidemiology of human infection appears complex since it relates to the route of transmission and a series of very different ecosystems that maintain the agent. Hence, the clinical presentation of the human disease is indicative of both the mode of transmission and often the source of infection. In Georgia, the literature and records describe predominately oropharyngeal tularemia acquired from drinking rodent contaminated water but local inquiry established that typhoid, pulmonary and influenza forms of the disease have also been diagnosed.

Geographic distribution

Tularemia outbreaks have been reported from many countries in the Northern Hemisphere, with particular prominence in Europe and Central Asia (Russia Federation, Kazakhstan, Turkmenistan, Turkey, Bulgaria, Rumania, Serbia, Sweden, Finland, Poland, Czechoslovakia, Hungary, Austria, Germany, Holland, Belgium, France, Portugal, Spain,), North America (USA, Canada) and Asia (Japan, China) [7-15]. Investigations of outbreaks and epizootics have identified a variety of animals and vectors responsible for transmission or maintaining the bacterium, depending on the region.

Molecular epidemiology studies have shed light on the geographic distribution of subspecies of *F. tularensis.* In North America, tularemia is primarily caused *by F. tularensis* subtype *tularensis* (type A), whereas subtype *holarctica* (type B) accounts for all known human cases in Europe and Asia and causes disease in North America[16-19]. Until the end of the 20th century, it was thought that type "A" occurred only in North America; however, it has been isolated in vectors (e.g., fleas and ticks) and rodents in Europe[20]. In Central Asia, *F. tularensis* subtype *mediaasiatica* has also been identified. While not associated with animal infection, *F. tularensis* subtype *novicida* has been identified as a cause of water-borne outbreaks in Europe (Spain), North American and a rare occurrence in Australia.[21]

Recent molecular epidemiology studies have shown the variable virulence of different genotypes. In general, type A is more the most virulent; however, this is solely due to strain A1b. A US study of genotypes has identified human infections with A1b to be the most virulent with the risk of infection estimated to be as high as 24%. Other genotypes have far less mortality risk (A1a (4%), A2 (0%), and type B (7%). No human cases of *mediaasiatica* have been identified.[22]

Incidence over time

Annual incidence rates of tularemia have limited value, as the disease typically occurs in outbreaks with relatively few sporadic cases being identified between outbreaks in endemic regions (the latter often due to ascertainment bias). Outbreaks may have hundreds of cases with no or few cases recognized in the years before and after resulting in highly unstable incidence rates over time. However some important incidence trends over time have occurred. Over the 20th century a dramatic drop in tularemia cases has occurred. The incidence peaked around World War II, reaching 100,000 annually in the former Soviet Union[23]. This high incidence was primary though to be due to exposure to food and water contaminated with rodent feces and carcasses. In the US, at its peak about 2,000 cases occurred just prior to World War II[24]. A large outbreak in Kosovo in 1999-2000 raised concern that *F. tularensis* may have been used as a biologic weapon.[25, 26]

The societal disruption associated with war was associated with the substantial incidence in the mid-20th century. The much lower current incidence is related to social order and the use of preventive behaviors to reduce exposure to infected arthropods (e.g., wearing long-pants), exposure to infected animals, water treatment and reduced rodent populations in food storage.

Modes of Transmission

Humans are infected by *F. tularensis* through inoculation, ingestion, and inhalation. Sources of infection occur from bites from various terrestrial and aquatic mammals, arthropod vectors, and ingestion of contaminated food and water. Aerosolization of feces and carcasses has also been implicated in outbreaks. For example, an outbreak in Sweden was caused by inhalation of *F. tularensis* from hay contaminated by vole feces.[27] In Martha's Vineyard, an outbreak was thought to be a result of inhalation of *F. tularensis* caused by a lawnmower shedding an infected rabbit carcass.[28] Inhalation is of particular concern given the more severe symptoms associated with it. The bacterium can remain viable for weeks in water, soil, and carcasses providing an opportunity for sufficient exposure to result in human infection.

Mammals are thought to be important in the environmental spread of *F. tularensis;* however, they are not thought to be a natural reservoir for the bacterium. Mammals transmit the bacterium to humans directly through bites and scratches as well as through arthropod vectors and contamination of water and food sources. The list of mammals with documented *F. tularensis* infections is over 200, however a small group of mammals are thought to be particularly important in the transmission to humans and sustainability of the organism in the environment (several types of rodents (e.g., voles, mice), hares, rabbits, ground squirrels, beavers and muskrats).[29]

Understanding the rodent population is critical in understanding the potential for human outbreaks. The size of the rodent population needs to be considered. Multiple human outbreaks have occurred following the increased density of the rodent population in a particular region. In addition, human outbreaks of *F. tularensis* have followed from outbreaks in rodents.

Several arthropods have been implicated in the transmission of tularemia. Depending on the region, mosquitoes (*Aedes, Culex, and Anopheles*), horse flies (*Tabanidae*), and ticks (*Ixodidae* and others) are established vectors. While arthropod vectors commonly are functional vectors of *F. tularensis* transmission, their role full in disease sustainability or potential to act as disease reservoirs is not clear. Previously, Olsufjev and Rudnev (9) defined major "eco-types" of disease foci that perpetuate *F. tularensis* respective to landscape, reservoir, vector and season (Table 3).

Risk factors for infection

Infection occurs among men and women of all ages. The incidence for men is over twice that for women; this is thought to be a result of outdoor activities that put them at higher risk. Hunters, farmers, landscapers and others working or recreating outdoors are at highest risk for infection. Another potential occupational risk factor is laboratory work; however, laboratory protocols have prevented infection in recent decades.[32] Table 1 lists the major factors describing epidemiological and ecological factors involved in tularemia emergence, transmission, and maintenance.

***F. tularensis* as a potential bioterrorism agent**

F. tularensis is a Category A select agent that can cause severe and fatal disease in humans if an aerosol containing as few as 10-100 colony forming units (CFU) of the bacterium is inhaled.[33] Several countries have weaponized *F. tularensis* for use as a possible biological weapon. Thus, *F. tularensis* is now considered a potential bioterrorism agent.

Tularemia in Georgia

Routine monitoring and investigation of tularemia in Georgia began in the mid-1940s and the first identified case of tularemia in South Caucasus was registered in Georgia in 1946. Since then two main regions with naturally occurring foci of tularemia have been identified: one in the mountainous Meskhet-Javakheti region[34] and another in the Kartl-Kakheti Valley[35]. The Meskhet-Javakheti focus covers the Ninotsminda, Akhalkalaki, Akhaltsikhe and Aspindza regions. The Kartl-Kakheti focus covers central and eastern part of Georgia. Epizootics of tularemia in the Meskhet-Javakheti focus were established during 1963, 1977, 1979 and 1980, 1984 and they were identified for the first time in the Kartl-Kakheti focus during 1956. The largest outbreak of tularemia with more than 300 cases was registered in the Ninotsminda region (village Gandza) in 1984 caused by drinking contaminated water. The last documented outbreak of tularemia

occurred in 2006[36]. The clinical presentations during these outbreaks involved mostly glandular and oropharyngeal forms. In several outbreaks, water was implicated as a source. Factors mediating the emergence of tularemia epidemics are outlined in Table 1. Historically cultures of *F. tularensis* were isolated from *Microtus arvalis* (common vole) and other rodents as well as from its fleas and ticks. Currently the pathogen repository at NCDC contains more than 90 isolates of *F. tularensis* that were isolated in Georgia during last 60 years. All of the Georgian isolates of *F. tularensis* that have been sub-speciated and belong to subtype *holarctica*.

During 2000-2008, 40 new cultures of *F. tularensis* were isolated by NCDC from natural focus areas in Georgia: 24 isolates from *D. marginatus* ticks, 1 - *H. sulcata* tick, 6 - *H. otophila* tick, 2 – *gamasid* mites, 3 - rodents, 4 – drinking water[37]. These observations suggest that tularemia is common in Georgia, and they emphasize the need to strengthen surveillance activities.
In the past preventive measures in Georgia included massive immunization of the population in natural foci, mainly this involved only the Meskhet-Javakheti region. The immunizations were conducted in this region every five years from 1956 until 1985 and involved 96,000 people in 1970. Since then, the numbers of infected individuals have decreased dramatically; however, no vaccinations were performed after 1985.

Table 2: Recent Isolation of *F. tularensis* in Georgia

Years	Strain	Source	Location	Isolation method
2009	*F. tularensis*	*D. marginatus*	Kaspi rayon	Culturing
	F. tularensis	*D. marginatus*	Kaspi rayon	Culturing
	F. tularensis	*D. marginatus*	Kaspi rayon	Culturing
2010	*F. tularensis*	*D. marginatus*	Kaspi rayon	Culturing
	F. tularensis	*D. marginatus*	Kaspi rayon	Culturing
	F. tularensis	*D. marginatus*	Kaspi rayon	Culturing
2011	*F. tularensis*	*D. marginatus*	Kaspi rayon	Culturing
	F. tularensis	*Hm. otophila*	Gori rayon	Culturing
	F. tularensis	*Hm. otophila*	Gori rayon	Culturing
	F. tularensis	*Hm. otophila*	Gori rayon	Culturing
	F. tularensis	*Hm. otophila*	Gori rayon	Culturing

Cooperative Biological Research, GG-18 highlights. The CBR project, GG-18 focused on an overall base-line study of *F. tularensis* in ticks and ectoparasites from 2008-2012. Molecular detection of *F. tularensis* target genes was performed in field samples with ectoparasite and rodent internals pool samples. In total, 271 ectoparasite pool samples were investigated on presence of *F. tularensis* genetic markers. Positive results were revealed in eight and undetermined in three DNA samples. Out of 138 rodent pool samples only eight DNA gave undetermined amplification. In total, 13 out of 18 isolate DNA samples were positive on both targets. From 11 samples (swabs and whole blood samples), no positive result was obtained.
In addition, Indel typing was completed. This allows for the characterization of isolates and relatedness grouping. Indel markers Ftind33, Ftind48 showed deletion for Georgian strains and LVS. Markers Ftind49, Ftind38, Ftind45, Ftind46, Ftind47, Ftind18, Ftind44, and Ftind50 revealed presence of insertion in Georgian isolates. Georgian strains together with LVS were typed as *F. tularensis* type B, *holarctica* and were placed in non-Japonica branch, East European subclade – B.19 by Svensson et al. and B.12 by Vogler et al.2009 (Vogler AJ, Birdsell D, Price LB, Bowers JR, Beckstrom-Sternberg SM, et al. (2009) Phylogeography of *Francisella tularensis*: Global Expansion of a Highly Fit Clone. J Bacteriol). According to Svensson et.al. Deletions for LVS should be appeared in Ftind50 (LVS subclade), Ftind33 (East European subclade), Ftind48 (*F. tularensis* type B, non-Japonica branch) and Ftind47 (*F. tularensis* type B, *holarctica*). In our data, Ftind50 and Ftind47 revealed presence of insertions in Georgian isolates and LVS strains that was not in accordance with article data. It worth to be mentioned that Ftind47 marker had

higher cross point (CP) and melt temperature (TM) compared to all other assays; it could be result of mutation in the region of interest. Ftind33 showed deletion in Georgian strains and LVS that could be considered as a logic result, but deletion appeared in SchuS4 strain DNA as well, that was used as a positive control in this test. In summary, the GG-18 project only looked at a diverse area of Georgia and was able to genotype only a few strains from the environment (non-human samples). Georgian strains along with the LVS strain, were typed as *F. tularensis* type B*, holarctica,* non-Japonica branch. In addition, the GG-18 project evaluated MLVA-25 and SNP typing on nine strains that were isolated from ectoparasite (Ixodae ticks) pools in Shida Kartli region. Isolated strains along with archival, LVS and SchuS4 reference DNA samples were typed using B.Br.012, B.Br.013 and 6 new SNP markers specific for *F. tularensis* spread in Georgia (B.Br.27-B.Br.32). These SNPs were discovered by whole genome comparisons and designed as canonical assays at Northern Arizona University (NAU) in previous studies (Phylogeography of *Francisella tularensis* subspecies holarctica from the country of Georgia, G, Chanturia, D. N Birdsell, M. Kekelidze, E. Zhgenti, G. Babuadze, N. Tsertsvadze, S. Tsanava, P. Imnadze, S. M Beckstrom-Sternberg, J. S Beckstrom-Sternberg, M. D Champion, S. Sinari, M. Gyuranecz, J. Farlow, A. H Pettus, E. L Kaufman, J. D Busch, T. Pearson, J. T Foster, A. J Vogler, D. M Wagner, P. Keim, BMC Microbiology 2011, 11:139).

The overall results of the GG-18 project, in relation to F. tularensis conclude that F. tularensis is in circulation of the same SNP group during last five years on a wide territory of the Shida Kartli region (as determined by isolation from only ectoparasites. This may reflect that this area could contain more active tularemia foci and emphasizes the need for a human seroprevalence study and preventive arrangements in the region, as proposed in this GG-19 project proposal.

Scientific Goals

This project is designed to provide the initial research and background information for the development of a comprehensive epidemiological surveillance project in Georgia and provide a base-line understanding of the geographic distribution of *F. tularensis* diseases in certain regions of this country. The research proposed will focus on at least two specific research priorities cited among the specific research priorities described in the CBEP Research Agenda (July 2011). In addition, we will perform basic research with all of the institutes with *F. tularensis*, which includes genotypic variations, bacteriophage research, and proteomic analysis of *F. tularensis* strains.

Aim 1: To study the seroprevalence of tularemia among exposed (people living in geographic areas known to be natural foci for tularemia) and not exposed healthy individuals and estimate risk factors for seropositivity.

Aim 2: To establish active surveillance for human tularemia clinical cases with the goal of increasing identification of the disease and collecting isolates from human cases for comparison to current environmental isolates and historical isolates.

Aim 3: To establish active surveillance for tularemia in the environment including small rodents and associated vectors and to identify the sources of tularemia outbreaks among humans. This effort includes inking environmental and animal cases with human cases.

Aim 4: To implement and evaluate diagnostic methods for tularemia and monitor patterns of antimicrobial resistance

Aim 5: To study isolated *Francisella tularensis* strains using DNA sequencing, proteomics analysis, and phage subtyping.

Technical Approach and Methodology

Aim 1: To study seroprevalence of tularemia among occupationally exposed and not exposed healthy individuals and estimate risk factors for seropositivity.

Rationale: The GG-18 project conclusions highly suggest that a human sero-prevalence study be conducted in Georgia in the *F. tularensis* active foci (as determined from ectoparasites). This study is incredibly important to determine area hot spots that may be attributed to human diseases caused by F. tularensis.

Approach: To address this hypothesis, a cross-sectional study design will be utilized for this aim. Study participants will be recruited from high-risk areas (hot spots) and other regions of Georgia for comparison. Study participants will be asked to complete an interview to identify risk factors for potential infection and provide a serum sample for antibody testing for *F. tularensis.* Up to 1000 patients will be screened in this Serosurvey over the three-year period.

Study Details:

- *High-risk areas:* High-risk areas identified with historical data and data obtained from the GG 18 project include Meskhet-Javakheti (Ninotsminda, Akhalkalaki, and Akhaltsikhe and Aspindza regions) and Kartl-Kakheti Valley (Eastern Georgia). Several villages historically known as hot spots for tularemia in these high prevalence areas will be the focus of data collection. Households in these villages will be randomly selected, and an adult individual in each selected household will be randomly selected for study participation. Eligible adults will meet the following criteria: 18 years and older, speak Georgian or Russian, lived in the region for at least 3 years consecutively, capable of providing informed consent, and willing to participate. Up to 500 patients will be enrolled in this study over the three-year period.
- *Low risk areas:* A comparison group of individuals with relatively low risk of infection and good health will be recruited. Individuals from low risk areas will be recruited from blood donors (State Blood Safety Program). Individuals will be selected from consecutive blood donors in these regions. Two further requirements need to be met: the number from each region will be pre-fixed, and frequency matching on age (in 10-year age groups) and gender will be required so that the distribution of high risk and low risk participants will be similar on age and gender. Up to 500 patients will be enrolled in this study over the three-year period.
- *Data collection:* Participants will be asked to complete an interviewer-administered questionnaire of risk factors for tularemia and provide a serum sample (high-risk group).
- *Blood samples:* Five ml venous blood will be drawn from consenting individuals recruited in high-risk areas and shipped to the laboratory of NCDC. All laboratory operational standards and biosafety requirements will be observed for sample shipment. NCDC is responsible for administering the State Blood Safety Program and within the framework blood donor samples are routinely sent to NCDC laboratory for ELISA testing on blood borne infections. De-identified samples will be used for tularemia serologic assays. Only an arbitrary linking code will be utilized to connect survey responses with blood test results. *F. tularensis* antibody tests in serum using microagglutination test (MAT) will be performed. The details of the laboratory test are discussed below. Any samples positive for *F. tularensis* antibodies will also be tested for *Brucella* antibodies to verify specificity of the test results for *F. tularensis* antibodies.
- *Questionnaire:* A standardized interviewer-administered questionnaire will be administered to all study participants by a trained interviewer. The questions will include demographic characteristics (e.g., age, gender, geographic region, occupation), exposure to risk factors for tularemia (e.g., outdoor activities, hunting), history of vaccination against tularemia in the past, prevention measures (e.g., use of DEET), recent illness, and utilization of medical care. For individuals who have been diagnosed with tularemia, clinical symptoms will be ascertained and documented.
- *Data Management and Statistical Analyses*: Each individual will be assigned a study identification number. Once data is linked, all identifiers will be dropped and only the study ID will be utilized for linking the questionnaire responses with the laboratory test

results. Data will be entered into SPSS and checked for quality and inconsistencies. The proportion of seropositive results (*Francisella tularensis*) will be computed separately for high risk and low risk individuals. Bivariate analyses will be conducted to compute the association between the seropositivity and other potential risk factors including age, gender, geographic region and type(s) of exposure. Multivariate analyses using logistic regression (outcome prevalence expected to be < 10%) will be conducted to identify independent predictors of seropositivity.

Aim 2: To establish active surveillance for human tularemia clinical cases with the goal of increasing the identification of the disease and collecting isolates from human cases for comparison to current environmental isolates and historical isolates.

Rationale: There has not been an active surveillance for human tularemia cases in Georgia in the past and all data has been obtained from ectoparasites to date. We suspect that several human clinical cases go undiagnosed and case studies are not accurate and need to be improved. We also hypothesize that *F. tularensis* case definitions can be improved to better diagnose this disease in humans.

Approach: To address this hypothesis, we plan to establish active surveillance for tularemia case finding in endemic areas in Georgia, such as Meskhet-Javakheti (Ninotsminda, Akhalkalaki, Akhaltsikhe and Aspindza regions) and Kartl-Kakheti Valley. The hospitals and primary care facilities in the tularemia hot spot regions will be involved. Regional referral hospitals and tertiary care hospitals will also be included in the study to recruit the patients potentially referred from above listed areas. The patients who will present to primary care centers or be admitted to the selected hospitals and meet the case definition for Tularemia (case definitions are described below) will be asked to participate in the study. Informed consent will be obtained prior to enrollment. Up to 1,000 patients will be enrolled in this study over the three-year period.

The following working case definitions will be used for different clinical forms of tularemia (Ulceroglandular, Glandular, Oculoglandular, Oropharyngeal, and Pneumonic):

a) *Ulceroglandular*,
Patient presenting with following clinical presentations:
- Fever > 38 C (oral temperature)
- Regional lymphadenitis (bubo)
- Skin lesion, which can present as ulcer, vesicular/pustule or popular lesions.

b) *Glandular*
Patient presenting with the following clinical presentations:
- Fever > 38 C (oral temperature)
- Regional lymphadenitis (bubo)

c) *Oculoglandular*
Patient presenting with following clinical presentations:
- Fever > 38 C (oral temperature)
- Severe unilateral conjunctivitis,
- Preauricular lymphadenitis

d) *Oropharyngeal*
Patient presenting with following clinical presentations:
- Fever > 38 C (oral temperature)
- Ulcerative-exudative stomatitis and pharyngitis, with or without tonsillar involvement
- Neck lymphadenitis

e) *Pulmonary and influenza*

Patient presenting with following clinical presentations:

- Fever > 38 C (oral temperature)
- Severe pneumonia
- Presentation from April to October of each year

The following set of laboratory investigations will be performed for each specific clinical syndrome of Tularemia. These will include the tests to identify *F. tularensis* and additional tests will be performed to identify other pathogens, which can present with similar clinical syndrome (for the detailed description of methods refers to Laboratory Investigations chapter below):

a) Ulceroglandular
   1. Bacterial cultures of the blood, lymph node aspirate and skin lesion swabs.
   2. MAT
   3. PCR of lymph node aspirates and skin lesion swabs
   4. Tests for differential diagnosis of other diseases presenting with similar syndromes: *Bartonella quintana, Bartonella henselae,* Syphilis, Anthrax, Rickettsiosis, HIV infection, Brucellosis, Leptospirosis, Q fever, Borreliosis, Ehrlichiosis

b) Glandular
   1. Bacterial cultures of the blood, lymph node aspirate
   2. MAT
   3. PCR of lymph node aspirate
   4. Tests for differential diagnosis of other diseases presenting with similar syndromes: *Bartonella quintana, Bartonella henselae,* Syphilis, Anthrax, Rickettsiosis, HIV infection, Brucellosis, Leptospirosis, Q fever, Borreliosis, Ehrlichiosis

c) Oculoglandular
   1. Bacterial cultures of the blood, lymph node aspirate, ocular lesion swab
   2. MAT
   3. PCR of lymph node aspirate and lesion swab
   4. Tests for differential diagnosis of other diseases presenting with similar syndromes: Adenoviral infection, Herpes simplex or Herpes zoster infection, Leptospirosis

d) Oropharyngeal
   1. Bacterial cultures of the blood, lymph node aspirate, oral lesion swab
   2. MAT
   3. PCR of lymph node aspirate and lesion swab
   4. Tests for differential diagnosis of other diseases presenting with similar syndromes: Diphtheria, EBV, CMV, Toxoplasma, HIV infection, Brucellosis, Leptospirosis, Q fever, Rickettsiosis, Borreliosis, Ehrlichiosis

e) Pulmonary
   1. Bacterial cultures of the blood and respiratory secretions (sputum, tracheal aspirate, bronchoalveolar lavage as appropriate) or pleural fluid if available.
   2. MAT
   3. PCR of sputum, respiratory secretions, or pleural fluid, as appropriate.

4. Tests for differential diagnosis of other diseases presenting with similar syndromes: Q fever, Viral pneumonia (influenza), Leptospirosis, HIV infection, Rickettsia, Ehrlichiosis

Based on clinical presentations and laboratory investigations, we will define presumptive and confirmed cases according to WHO Guidelines on Tularemia: [38]

- **Presumptive cases** - suggestive clinical symptoms and a clinical sample that tests positive for tularemia by DNA detection or a single positive serum.

- **Confirmed cases** - the recovery of an isolate and identification of the culture as *F. tularensis,* or fourfold difference in titer of paired serum specimens.

All presumptive and confirmed cases will be documented in Electronic Disease Surveillance System (EIDSS) and the GIS database. Both presumptive and confirmed cases will be used to determine the incidence of tularemia.

Aim 3: To establish active surveillance for tularemia in the environment including small rodents and associated vectors and to identify the sources of tularemia outbreaks among humans and linking environmental and animal cases with human cases.

Rationale: We will define the types of endemic foci recorded in Georgia based on a historical analysis of strain database information and to select new representative areas that include the known three types of foci: steppe, meadow field and forest types (Table 3). The data that make up this hypothesis comes from the GG-18 project, which provided a broad base of information on ectoparasite data. However, the GG-18 data did reveal that additional environmental samples would need to be conducted, especially in endemic foci for this disease, and, environmental collection needs to coincide with human clinical studies. To this end, we will conduct environmental surveillance for *F. tularensis* in areas where historical outbreaks have been recorded and extensive field investigation undertaken of the environment (Figure 1).

Figure 1: Historical map showing the prevalence of Tularemia (highlighted in blue) in the Republic of Georgia.

Approach: Tick surveillance and rodent surveillance will be conducted to determine the current prevalence of *F. tularensis* in vectors and reservoirs to determine their impact on human disease risk. A historical review of isolation rates appertaining to the environmental sources of the NCDC database of strains will be undertaken to optimize recovery of *F. tularensis* from rodents, ticks, fleas, and related materials.

Table 3: Classification of tularemia foci in former USSR (from Olsufjev & Rudnev 1960)

Kind of foci	Main reservoir	Tick involved	Season of outbreaks
Meadow-field type	*Microtus arvalis*	*Dermacentor pictus*	Winter
Steppe (ravine) type	Voles, mice, hamsters, hares, etc	Mainly Dermacentor merginatus	Autumn and winter
Forest type	Red vole (*Clethryionymous glareolus*), forest mice, hares	*Ixodes ricinis, I. frianguliceps*	Tick season
Floodland-swamp type	Water rates	*Dermacentor, Rhipicephalus, Ixodes* sp.	Season of water-rat hunting
Foothill type	Water rats (voles, etc)	*Ixodes apronophorus*	Summer
'Tungai' type	Hares, gerbils, mice, and muskrats	*Rhipicephalus pumilio*	Season of hare hunting

There have been 13-recorded outbreaks of tularemia in five foci in Georgia. The largest outbreaks have been recorded from the central and south-central regions of Georgia: Meskhet-Javakheti (Ninotsminda, Akhalkalaki, Akhaltsikhe, and Aspindza regions) and Shida-Kartli regions. These outbreaks included suspected water-borne and rodent-borne transmission. An additional historical outbreak occurred in the village of Zhemo Kedi, Kakheti region, in eastern Georgia and involved children and the threshing of wheat. Table 4 (below) summarizes the temporal distribution of 442 isolates of *F. tularensis* in Georgia and the source data from rodents, arthropod, and other samples.

Table 4: Temporal analysis summary

Year	*M.arv*	*M.soc*	*P.vole*	*A.syl*	*shrew*	*A.terr*	*M.mus*	other	*D.marg*	*Ix.ric*	*Haem*	*Gamaz*	other	*Cteres*	Bird	water	wheat	**Total**
1950	1	1	–	–	–	–	–	–	1	1	–	1		2	–	–	–	***7***
1960	16	2	–	1	–	–	2	2			–	1	1	5	–	–	–	***29***
1970	57	–	3	4	3	1	–	2	5	7	1	7	1	19	1	–	–	***120***
1980	50	7	–	2	4	2	1	1	32	6	16	3	3	11	–	2	–	***137***
1990	21	–	–		–	3	–	–	47	12	4	2		13	–	–	2	***102***
2000	3	–	–	1	–		–	–	30		7	4	1	1	–	4	–	***50***
Total:	***148***	***10***	***3***	***8***	***7***	***6***	***3***	***5***	***115***	***29***	***28***	***18***	***6***	***51***	***1***	***6***	***2***	***442***

The NCDC database of historical isolates of *F. tularensis holartica* will be subject to a more detailed review of isolation rates from the field study records to determine the effective denominator that may indicate carriage and persistence in the approximately 60 different locations sampled since the 1950s. This database will also be used to map using GIS the approximate distributions of the 440 source related isolates

In conjunction with Dr. John Lee's Vector Surveillance group at USAMRIID (and using the IACUC approved protocols through USAMRIID), the team, approximately four times per year, will conduct field expeditions in the *Francisella tularensis* focus areas. Field trips will last approximately 15 days per trip. Ticks will be collected off livestock (reference of use in Georgia) or by using tick drags and standard classification/taxonomy will be used to identify the ticks to species, followed by pooling according to species, location, and collection date. DNA from the tick pools will be isolated and real-time PCR analysis will be performed to screen the pools for the presence of *F. tularensis* (see Laboratory investigations, section g). Rodents will be collected using Sherman live-catch traps set in fields, in, and around houses. Collected rodents will be identified to species, checked for ectoparasites, and then processed to obtain blood as well as spleen and kidney tissues. Blood, tissue, and ectoparasite samples will be tested by real-time PCR *for F. tularensis* (see Laboratory Investigation). In total, it is estimated that 1,000 samples (ticks, tissue, etc) will be collected and processed per expedition, with a total estimate of about 4,000 specimens per year during the three-year period.

For all presumptive and confirmed cases, an epidemiological investigation will be carried out. The patient will be interviewed and case investigation report form completed. Information collected on the case investigation form will include general patient information (e.g. age, sex,

residence, etc) as well as collect information about the patient's activities (e.g. hunting), potential exposures (e.g. tick or mosquito bite, contact with rodents, etc), and location of potential exposure. Tick surveillance and rodent surveillance will be conducted around a patient's residence and farm (or work site), if applicable. For all presumptive and confirmed cases, an epidemiological investigation will be conducted to determine if a link exists between the human case(s) and those pathogens found in the vectors and reservoirs. Methods from Task 1 will be applied here. Genetic analysis will be used to link human and environmental isolates, see Aim 6.

Geographical Information System (GIS) analysis will be used to determine a risk assessment for exposure to environmental and animal sources *of F. tularensis*. This will be accomplished by using GIS software systems, specifically ESRI ARC Map/Editor. Global Positioning System (GPS) handheld units will be used to record the coordinates of the collection sites, number and species of arthropods collected, and collection site habitat data. The final output of the GIS analysis will be risk assessment maps. The risk assessment maps will be composed of multiple biotic and a-biotic layers, to include: current vector distributions broken down by species determined from the active surveillance, disease burden associated with the vectors collected and tested during the active surveillance, road and waterways, vegetation, elevation, and any historical data pertaining to Tularemia (Figure 1) and current data from imagery and weather services

Aim 4: To implement and evaluate the diagnostic methods for tularemia and to monitor antimicrobial resistance.

Rationale: Historically, culturing *F. tularensis* been difficult as it is a fastidious, slow-growing organism. Blood cultures are often negative, most likely because the initial infection has not progressed to the blood stream. The collection of multiple sample types (e.g. swab of ulcer, lymph node aspirate, and blood) from a patient will increase the likelihood of isolate recovery. In this protocol, we hypothesize that by taking a number of additional steps will be taken to increase the likelihood of culture recovery on agar media. These include collection and transport methods to provide for maintenance of viable organisms, culture initiation within 24 hours of the sample being taken from clinical cases, storage of environmental samples at -80^{o}C to preserve viable organisms, increased incubation times of primary cultures (7-10 days) and the use of antibiotic media to allow for isolation of *F. tularensis* in the presence of other organisms. Typically, mice have been used to isolate *F. tula*rensis from contaminated samples as they are highly susceptible to *F. tularensis* and act as a filter to amplify this organism in the presence of other non-pathogenic organisms. The use of antibiotic media to isolate *F. tularensis* from contaminated samples alleviates the dependence on mice for the isolation of *F. tularensis*. Through this rationale, we proposed to work with our collaborators and our expertise in bacteriology to isolate *F. tularensis* from human clinical samples for the first time without the use of mice for propagation.

Approach: The following approach will be taken to complete these study objectives in Aim 4:

Patient recruitment and evaluation: All tularemia-suspected patients recruited in the study will be tested by all appropriate laboratory methods as described in this document. The gold standard for laboratory confirmation will be either a four-fold rise in antibody titers using paired samples or isolation of *Francisella tularensis* in cultures. Sensitivity and specificity of other diagnostic tests including PCR will be calculated and compared to the gold standard. While the primary purpose of testing for a range of potential co-infections is related to research ethics, these studies will provide knowledge about the causes of infections among those negative for *F. tularensis*. Additionally, the presence of co-infections will provide insight into this potential risk factor for tularemia infection.

Sample collection and transportation: Clinical samples will be collected and shipped to the laboratories of NCDC observing all laboratory operational standards and biosafety requirements for sample collection and shipment and using new methods published by the Booz Allen Hamilton Biosafety and Biosecurity for Sample transport. The specimens will be delivered to the laboratory within 24 h under the standard conditions; environmental samples will be kept at -80C in the field

and will be delivered to the laboratory periodically (based on the project schedule) under the standard conditions. Transport temperature conditions to the laboratory will be those recommended by the WHO Guidelines in order to ensure organisms remain viable during this process. All samples will be labeled clearly with all required information (name, ID, source, site, date etc.)[39]

Specimens will be considered acceptable for the various forms of human Tularemia syndromes as specified:

- Whole blood - for all clinical forms of illnesses. Two samples of venous blood will be obtained, preferably from separate sites and 30-60 minutes apart, comprising in total 20-30 ml, into bottles of a conventional aerobic blood culture system.
- Serum - for all clinical forms of illnesses. A first specimen will be collected as early in the course of infection as possible, followed by a second specimen taken in the convalescent period (at least 14 days later).
- Respiratory secretions/pleural fluid – for pneumonic form of illness. Bronchial/tracheal washes or aspirates, sputum, transthoraxic lung aspirates, or pleural fluid. Specimens will be collected in screw-capped containers and transported to the laboratory as quickly as possible.
- Swabs – of visible lesions or affected areas will be collected for Ulceroglandular, Oculoglandular and Oropharyngeal forms of illness. For culture, a rayon-tipped plastic applicator and a tube-containing agar with charcoal will be used.
- Aspirates – from lymph nodes or lesions for Ulceroglandular, glandular, Oculoglandular and Oropharyngeal forms of illnesses will be collected with sterile needle and syringe and transferred aseptically into a sterile tube.

Specimens acceptable for the animal and environmental samples:

- Mammals – serum, aspirate, autopsy materials;
- Arthropods – ticks, fleas, mosquitoes;
- Environmental samples – soil, water, and rodent feces will only be collected in the context of an epidemiological investigation that indicates these as the potential exposure sources.

Bacteriology: Samples will be screened bacteriologically, by plating them on Cysteine heart agar enriched with chocolatized red blood cells (CHAB) which is a practical medium for culture of *F. tularensis* as the organism displays characteristic morphology and grows well on this media; Plating on Cysteine enriched Chocolate agar (CA) or Sheep Blood Agar (SBA) will be used to identify any possible existing co-infection. CHAB medium supplemented with antibiotics (CHAB-A) has been shown to significantly improve (81%) recovery rates from tissue sources contaminated or overgrown by other flora.[40] In specimens where the presence of mixed flora is likely, including respiratory and Oropharyngeal samples CHAB-A will be used For any primary samples were other bacterial growth is observed when the sample is cultured on non antibiotic CHAB media, the samples will be re-plated on antibiotic containing CHAB media for isolation of *F. tularensis.*

For blood culture, the automatic blood culture system will be used. This will allow for the amplification of small numbers of *F. tularensis* organisms in blood samples prior to culturing on media. Aliquots from positive blood culture bottles will be incubated on CHAB. Blood culture bottles will be maintained for 7-10 days.

All inoculated culture plates will be incubated at 35-37C for 7-10 days at 5% CO_2 and checked daily for growth. A portion of the clinical samples will be stored for any future testing (including additional culture work) that might be required. Bacterial isolates will be identified as *F. tularensis* using standard criteria, including morphology and biochemical tests, as recommended by the CDC.[41]

Differentiation of species and subspecies of *F. tularensis* will be based on growth characteristics, biochemical bio typing (Table 5) (Cysteine requirement; carbohydrate fermentation: maltose, sucrose, D-glucose, glycerol; citrulline ureidase production; oxidase production; H_2S production on TSI);[42] and molecular biotyping.

Table 5: Discriminating characteristics of *Francisella* species and subspecies

Characteristics	***F. tularensis* subspecies**				***F. philomiragia***
	tularensis	***holarctica***	***mediaasiatica***	***novicida***	
Cysteine requirement	+	+	+	-	-
Maltose fermentation	+	+	-	+	+
Sucrose fermentation	-	-	-	+	+
D-glucose fermentation	+	+	-	+	+
Glycerol fermentation	+	-	+	+	-
Citrulline ureidase production	+	-	+	+	nd
Oxidase production	-	-	-	-	+
H_2S production	+	NA	NA	NA	NA

Note: "+" positive";-"negative; "nt" not tested; "NA" not available.

Antigen detection: Antigen detection is useful for either direct identification of *F. tularensis* in clinical/environmental specimens (direct fluorescent antibody (DFA)) or for confirmatory identification of isolates recovered in culture (slide agglutination or DFA).

- DFA staining using FITC-labeled rabbit antibody directed against whole killed *F. tularensis* cells (rapid assay for identification of *F. tularensis* in primary specimens or for confirmation of recovered isolates) will be used.[43]
- Slide agglutination will be used for the rapid conformation of recovered isolates. Slide agglutination assay is based on the interaction between high-titer anti-*F. tularensis* antibody and *F. tularensis*-specific antigens. A positive slide agglutination (clumping of cells) test is confirmatory for *F. tularensis.*

Serology: Serology is commonly used for confirmation of tularemia. Antibody responses against *F. tularensis* are generally detectable in patients 10-20 day's post-infection.[44]

- Microagglutination and/or tube agglutination, the standard serological tests for determining the presence of antibody to *F. tularensis*,[45] will be used in paired human serum specimens and/or in mammalian serum samples for conforming exposure (infection and vaccination) and for Sero-prevalence studies.
- Tube agglutination: house-made diagnostic antigens and formalin-fixed, safranin-stained *F. tularensis* cells will be used according to CDC SOP (Fort Collins, CO) respectively. The tube agglutination test, which is currently used in the NCDC lab, will be used initially to validate the microagglutination assay and a cut-off value for diagnostic purposes in Georgia will be determined.
- Enzyme-linked immunosorbent assay (ELISA). We will be using ELISA methods as described elsewhere by N. Chitadze et al.[46]

Laboratory Diagnostics to rule-out similar infections and presentation: The following methods will be utilized to identify the pathogens during differential diagnostic work up of patients with suspected tularemia. ELISA tests will include: Bartonella quintana IgM, Bartonella quintana IgG, Bartonella henselae IgM, Bartonella henselae IgG, Rickettsia IgM, Rickettsia IgG, Brucellosis IgG, Brucellosis IgM, HIV antibodies, Leptospirosis IgM, Leptospirosis IgG, Coxiella burnetii I IgM, Coxiella burnetii I IgG, Coxiella burnetii II IgM, Coxiella burnetii II IgG, Borrelia IgM, Borrelia IgG, Ehrlichia IgM, Ehrlichia IgG, EBV (VCA IgG), EBV (VCA IgM), EBV (EBNA IgG), EBV (EA), CMV (IgM), CMV (IgG), Toxoplasma IgM, Toxoplasma IgG. Bacteriology: Bacillus anthracis culture from lesions, Corynebacterium diphtheria culture from pharynx. PCR: Bacillus anthracis PCR

from skin lesions, Herpes simplex 1&2 PCR from eye lesions, Herpes zoster PCR from eye lesions, Adenovirus PCR from eye lesions, Influenza PCR from respiratory samples.

Antimicrobial susceptibility: Antibiotic susceptibility of the recovered isolate to the following antibiotic agents using CLSI breakpoints will be performed using the E-test method[47]: Streptomycin, Gentamycin, Tetracycline, Doxycycline, Chlorampenicol, Rifampicin, Ciprofloxacin, Levofloxacin, and Moxifloxacin.

Aim 5: To study isolated *Francisella tularensis* strains using DNA sequencing, proteomic analysis, and phage subtyping.

Rationale: The GG-18 CBR project (2008-2011) made great strides in characterizing a limited number of F. tularensis strains that were isolated from ectoparasites. The GG-18 project data suggested that there are several more questions that need to be answered about *F. tularensis*. The first is that the molecular characterization of F. tularensis strains is incomplete and there is an incomplete picture not only from human isolates (never been completed), but that there is a tremendous genetic diversity in *F. tularensis* strains from the limited number tested. This proposal will look to complete the genotyping picture in Georgia.

In addition, proteomic analysis of *F. tularensis* isolates under the GG-18 project indicates differences between selected isolates of *F. tularensis* on a proteome level. This knowledge is very important, since differences on a genomic level quite often are "silent" and not expressed. It has been shown by decades of research with other pathogens that most of the in-consistently differentially expressed proteins are virulent factors and their knowledge of them could pave ways for better diagnostics and identification schemes. The same is true for the potential application of this potential data for the protection of civilian and military personnel. Under GG-18, the project team was able to increase the capabilities of the Georgian Institutes with the first real, full-scale (from identification of the proteins on 2-D gels) proteomic research in Georgia. We anticipate that continued research in this area will reveal a list of differentially expressed proteins, which will lead to future studies and projects and provide a better understanding of *F. tularensis* physiology and virulence.
Finally, until the GG-18 project, nowhere in the world has a bacteriophage been infective to and react to F. tularensis. The Eliava institute, over three years under GG-18 research, has isolated several key non-stable and reactive *F. tularensis* bacteriophage. This is hallmark and cutting-edge research, to, for the first time, isolate, and characterize stable *F. tularensis* bacteriophage. It is our hypothesis that this approach will yield such results.

Approach: We will take a multi-fold approach to address the hypotheses in this aim:

Molecular diagnostic of Francisella tularensis in clinical / environmental samples: For DNA extractions, some samples (sputum, bronchial and tracheal washings) will be specifically pre-treated with N-acetyl-L-Cysteine or other specialized reagents to liquefy the samples; and some tissue samples will be incubated in Qiagen tissue lyses buffer (ATL) at 56^{O}C until fully lysed, followed by DNA purification using Qiagen DNA Mini Kit. DNA extraction from ground tick and rodent samples will be performed using Qiagen DNA Blood and Tissue Mini Kit.

Real Time PCR analysis will be accomplished using Idaho Technology Kits: *F. tularensis* Target 1 and Target 2; and will be performed for all clinical samples, while environmental samples, such as tick pools and rodent tissues will be screened using the Real Time PCR multi-target primer set ISFtu2, tul4, and iglC[48].To avoid cross reactivity with ectoparasites endosymbionts, positive tick pool samples will be amplified using a 16S ribosomal RNA gene primer set, purified, and then sequenced and compared to other bacterial genomes in the NCBI database by BLAST analysis.[49,50]

The same approach will be used for positive clinical DNA samples to determine the species / subspecies of the disease agent. As it has already been determined, *the F. tularensis* present in

Georgia belongs to the subspecies *holarctica* (type B), but very rarely in immunocompromised patients, disease can be caused by *Francisella tularensis* subsp. *novicida* or by *F. tularensis* subsp. *tularensis* (type A). While *F. tularensis* Type A and type B are easy to distinguish using routine PCR analysis, *F. tularensis* subsp. *novicida* can be confirmed only after sequencing. For clinical samples, PCR positivity will be compared with culture positivity, to determine the sensitivity of direct plating for identification of *F. tularensis.*

Molecular characterization of newly isolated F. tularensis strains: Newly isolated strains from environmental and human exposures will be characterized using different typing methods established at the NCDC of Georgia. Canonical SNP markers for *F. tularensis* B.Br.027[51] and MLVA-25 markers F.t.M3 and F.t.M4[52] will be used to characterize strains isolated. Besides, PCR positive clinical and tick/rodent DNA samples can be directly characterized before culture isolation with a rapid MLVA typing system using variable F.t.M3, F.t.M4 markers. The SNP typing methodology will be applied on the Real Time PCR cycler using the Sybr Green master mix, while PCR-based MLVA technique will be performed on Beckman Coulter Genetic Analysis System CEQ-8000.

All typing results will be added to the existing *F. tularensis* database and analyzed in compliance with phylogenetic, geographical, and epidemiological data. The DNA sequence of conserved *F. tularensis* housekeeping genetic markers (i.e., 16s RNA) will be determined at the CPHRL and compared with archival and *in silico* database sequences in order to augment diagnostic validation of species and subspecies determination. In addition, DNA sequencing of known molecular determinants conferring phenotypic variation within the *F. t. holarctica* subspecies will be performed to yield insight into the nature of molecular and phenotypic diversity within and across environmental and human-derived specimens.

<u>*Proteomic Studies:*</u> Different isolates of tularemia along with the reference live vaccine strain LVS will be cultivated in Heart Infusion Broth (HIB) medium until they will reach logarithmic growth. Bacterial cells will be collected by centrifugation, lysed in a buffer for 2-D electrophoresis, and incubated for 10 minutes at 95C. Lysates will be tested for sterility and further used in biochemical studies. The 2-D electrophoresis system from GE HEALTHCARE will be used. Iso-electrofocusing (IEF) will be carried out according to approved protocols that were developed under the CBR GG-18 project. In the pilot studies, we will test mainly two types of IEF strips: linear and non-linear strips with pH 3.0-10.0. SDS electrophoresis will be run using a gel apparatus system. Therefore, on one gel the strip with proteome of LVS tularemia samples will be loaded. Another five gels will be loaded with samples from the strains of interest. Gels will be silver stained without gluteraldehyde (in order not to interfere with mass spectrometry (MS) analysis) and images will be analyzed with 2-D Platinum software (GE Healthcare) to reveal differentially expressed bands. Only spots with more than two-fold of differences and showing consistent changes will be excised and subjected to MS analysis.

<u>*Comparative proteomic profiling of culture filtrate proteins of different Francisella tularensis strains*</u>: Bacteria secrete various substances as a means of coping with and surviving in hostile environments. Secreted proteins play important roles in bacterial survival strategies such as provisioning of nutrients, detoxification of the surrounding milieu, cell-to-cell communication, and modulation of host cell responses, and host-tissue colonization. They are also recognized as major virulence factors in the pathogenic process.[52] Therefore comparative characterizations of Georgian isolates of *Francisella tularensis* from this point is also of great importance. Selected isolates of *F. tularensis,* compared to the reference strain, will be cultivated in chemically defined Chamberlain medium. Culture filtrate proteins will be collected as described in the referenced methods.[53] 2-D electrophoresis and data analysis will carried out as described above for the tularemia proteome experiments and as used in the GG-18 project.

<u>*Phage typing as an additional tool for identification:*</u> Selective phage typing will be used as an additional differential diagnostic effort. This component will include subjecting aliquots of clinical sample material to a diagnostic panel of diverse selective phages to aid in identifying the

presence of pathogens (*F. tularensis*, as well as Brucella for testing cross-reactivity) are included in the differential diagnostic regimen. Despite many efforts of scientists from different countries, there is still no report on the isolation of bacteriophage specific to *F. tularensis*. A limited number of documents presented by Russian scientists described isolation of Francisella bacteriophages, but no paper explains the stability and reliability of isolated phages. The Eliava Institute scientists have been working on the isolation of bacteriophage against vaccine strains of *F. tularensis* in the framework of the GG-18 project. To date, two bacteriophages with host bacterial strains from *P. aeruginosa* reveal lytic activity to the vaccine strain of Francisella, although the data need more detailed investigation and validation.

<u>*New Phage Discovery:*</u> Specimens, lytic for *F. tularensis* obtained from animal or environmental samples will be used to determine phage existence. Bacteriophages will be isolated by using standard enrichment techniques (Adams, 1961). A mixture of environmental samples (minced or grained) and appropriate bacterial cultures with different ratios will be incubated at relevant temperature for 12-24 hours. Suspensions will be centrifuged, filtered, and spotted on the lawn of corresponding bacterial strain. The plates then will be incubated during next 12-24 hours to detect clear zones of phage activity. The vaccine strain *F. tularensis* LVS (not a select agent) will be used for experiments for phage isolation. The agar overlay method will be used to enumerate formed phage plaques. The same approach will be used for phage isolation from the patient's blood sera. After several rounds of passages of isolated phage(s) on corresponding host bacterial strain, pure phage clones will be obtained. Isolated phages will be studied in detail, particularly; we will study the morphology of phage particles, physiology (one-step growth properties), DNA characteristics, and structural proteins. Phages in high titer will be prepared and tested against bacterial collection of *F. tularensis* of the NCDC for determination of host range and specificity. Phages will be used for typing, as an additional test for diagnostic of bacterial strains of *F. tularensis*. The data will be evaluated on specificity and sensitivity and compared to the gold standard method for diagnostic of tularemia.

Expected Results

This project will:

- Estimate the public health burden of tularemia in Georgia
- Strengthen early identification and control capacity by:
    - Establishment, evaluation and validation of different diagnostic modalities
    - Fortification of the public health institutional and laboratory infrastructure and its response to tularemia outbreaks
    - Improving recognition of the disease and detection of risk factors for outbreaks
    - Strategic planning for disease control efforts to prevent outbreaks
- Describe the genotypic and phenotypic characteristics and antimicrobial resistance patterns
- Describe risk factors for transmission of tularemia
- Increase awareness among physicians, public health professionals as well as the general population regarding tularemia
- Implement and evaluate diagnostic methods for tularemia
- Provide better recognition and treatment will reduce the burden of disease and its concomitant socioeconomic impact. Enhanced surveillance will facilitate development of cooperative disease control efforts in the region to further anticipate outbreaks and contain transmission.

Human Subjects Considerations

As with all research projects we conduct, careful attention is being paid to maximize the benefit of the research to both the participants and community while minimizing harm. The Belmont Report is the framework utilized in developing study methods with constant focus on ethical considerations. Informed consent will be sought from all participants. The informed consent process will provide clear information about the study and emphasize participation is voluntary.

In Georgia, making sure participation is voluntary is critical, as saying no is culturally difficult at times. Participants will also be clearly told that they can stop participation at any time. We expect substantial interest in participation given the benefits the study provides to participants.

The study has direct benefits to participants, as they will receive high quality laboratory tests to determine tularemia infection and potential co-infections. Such laboratory tests have limited availability in the country and typically are expensive. For those who have a negative test for *F. tularensis*, tests for co-infection will be valuable to provide an accurate diagnosis. This step is critical to maintain positive relations with participants and the community. To test only for *F. tularensis* and provide no insight into the cause of disease for those who are negative could results in a sense of being "used" for research by investigators. The community will benefit by knowing the existence of tularemia and the types of infections that are difficult to differentiate from tularemia.

All data will be kept by the NCDC in protected computers in protected offices. Only participants (and their physicians if consented) will be provided individual laboratory results. Confidentiality of information will be a high priority to protect participants for any form of discrimination.

NCDC will provide communities in the regions the study is conducted a report of the general findings without individual identifiers. This report will include information on the risk factors for infection identified, the number of people infected, and general information on protective measures.

IRB review and hospital sites for sample collection from Human Subjects: As of January 2012, the main hospital system is being overhauled in Georgia. During this process, the actual hospital may be moved, closed, consolidated, or replaced. Therefore, final hospital site locations have not yet been determined for this project. Because of this issue, we will only describe the process for sample collection (below) and transport. Individual hospital locations will be added to this Form A as an appendix for the purposes of completing the facility assessments of a Biosafety and Biosecurity Review. Samples, once taken from consenting human subjects will be transported to the TADR network of ZDL's and LSS's laboratories for all testing and diagnostics. The hospitals only serve as a means to collect the specimen and receive informed consent. Individual IRB protocols will be written, reviewed, and approved once preliminary approval is obtained to begin the project. A separate biosafety and biosecurity review for the clinical protocols will be handled separately between clinical protocol completion and IRB approval.

Project Management

This project focuses on *F. tularensis* and will be undertaken at both the CPHRL and at NCDC in Tbilisi, Georgia. The institute Director will appoint a Project Manager for the work to be performed at the institute. This work will be supported by an institute specific budget to perform the tasks outlined in this project description. In addition, there will be an overall Project Coordinator to serve as a point of contact for CBEP on the activities engaged under this project. The main task of the Project Coordinator is to ensure that all communications amongst the parties are supported and that coordination of data between the human and animal studies is working. The duties of the Project Coordinator are listed below.

1. Project Reporting
   - Consolidation of quarterly reports from participating institutes
   - Preparation of a short executive summary of quarterly reports
   - Leadership in preparation of project presentations and abstracts for conferences and international meetings
   - Facilitation of publications in peer reviewed journals

2. Scientific Leadership

- Regular communications with U.S. Collaborators
- Participation in Quarterly project calls (and monthly if time permits)
- Organization of local meetings of the Georgian project participants to discuss the project progress
- Participation in international conferences and meetings.

Meeting Goals and Objectives of CBR Program

This proposal addresses three CBEP goals:

1. strengthening dangerous pathogen detection and response networks
2. enabling discovery of the diversion or accidental release of biological materials
3. integrating host nation scientists and institutes with expertise in research and production into the international scientific community

The proposed study is important considering that *F. tularensis* species remains a significant veterinary and medical threat in Georgia. From a public health perspective, the development and application of more advance diagnostics could lead to a superior disease monitoring capability for the institute and function to enhance disease control efforts in Georgia. Detection and surveillance of these pathogens in natural foci areas will also permit an integrated understanding of pathogen circulation in the region. The design of the program is such that it requires significant interaction with collaborators and scientists and institutes in the form of training, participation in U.S. scientific workshops and conferences and working in close contact with collaborating scientists in their laboratories.

Biosafety and Biosecurity Overview: All of the targeted bacterial pathogens are Class A agents of high bioterrorism importance and their handling is normally conducted under BL-3 safety conditions. All of the studies proposed in this application will adhere to the biosafety recommendations contained in the 5th edition of the Biosafety in Microbiological and Biomedical Laboratories (BMBL) Manual published jointly by the CDC and NIH (relevant text from the BMBL Manual is reproduced below for each pathogen). Samples may be processed in the field in mobile laboratories of the NCDC or in the NCDC's field stations. When the microbiological examination of those samples suggests that a targeted organism may be present, the samples will be transferred to the main laboratory at the NCDC for subsequent identification and processing using the biosafety recommendations for the targeted bacteria (From the 5th edition of the BMBL). Biosecurity: all samples will be kept in locked laboratories and locked refrigerators. Sample logbooks will be maintained and all samples and cultures will be kept under standard security measures as defined by CBEP.

F. tularensis: BSL-2 practices, containment equipment, and facilities are recommended for activities involving clinical materials of human or animal origin suspected or known to contain F. tularensis. Laboratory personnel should be informed of the possibility of tularemia as a differential diagnosis when samples are submitted for diagnostic tests. BSL-3 and ABSL-3 practices, containment equipment, and facilities are recommended for all manipulations of suspect cultures and animal necropsies. Preparatory work on cultures or contaminated materials for automated identification systems should be performed at BSL-3. Characterized strains of reduced virulence such as *F. tularensis* Type B (strain LVS) and *F. tularensis* subsp. *novicida* (strain U112) can be manipulated in BSL-2. Manipulation of reduced virulence strains at high concentrations should be conducted using BSL-3 practices.

Safety Issues: Safety at sample collection sites will be ensured by the use of standard personal protective equipment (PPE) as and where appropriate. We will use the following equipment: Tyvek coveralls, boots (12 in high), N95 respirators, safety goggles, disposable hats, and double gloves (inside pair taped to the sleeves of the coverall). Staff at the collection sites have been using PPE for many years, and are skilled in the safe handling of potentially infectious material. Samples for

PCR analysis will be handled appropriately in Eppendorf tubes and heat inactivated at the earliest feasible time after sample collection and, as a result, the samples will be safe and non-infectious.

Pathogen	Biosafety Practices	Primary Barriers and Equipment	Facilities and Secondary Barriers
F. tularensis	- Standard Microbiological Practices - Limited and controlled access - Biohazard warning signs - "Sharps" precautions - Biosafety manual / SOP - Decontamination of all waste - Decontamination of laboratory clothing before laundering - Baseline serum	- Class I or II BSCs or other physical containment devices used for all open manipulation of agents - Protective laboratory clothing; gloves; respiratory protection as needed	- Laboratory bench and sink - Autoclave - Physical separation from access corridors - Self-closing, double-door access - Exhaust air not re-circulated - Negative airflow into laboratory

Approximate Budget

Additional details of the budget are in the Form B. The approximate budget for the Georgian Institutes for this project is $ $ 1,656,044.73 over 3 years.

Relevance to DOD Program Objectives

- Knowledge of regional pathogens. This study will increase the knowledge of the epidemiology of *F. tularensis* in understudied geographic areas of natural and new foci's in Georgia and test hypotheses on the environmental conditions that promote tularemia outbreaks in-country.
- Gainful employment of scientists. This project will provide gainful employment for scientists working with especially dangerous pathogens.
- Transparency and integration. This project will promote transparency and integration of Georgian scientists into the global science community through participation in international conferences and publication of scientific papers in international journals.
- DOD has a significant requirement to establish the Baseline of Pathogenic Organisms in the Environment. This enhanced surveillance program resulting from this project will generate new information on important especially dangerous pathogens which will be mapped and described in detail.

Relationship to Other on-going, planned, and recently completed CBEP Projects

This study will serve as follow-on to the GG-18 project and links well with the ongoing Vector Surveillance studies in Georgia. The previous project, GG-18 demonstrated the presence of *F. tularensis* in Georgia and previously collected and new isolates and thousands of samples that could still be used on this project. CBEP encourages work to collect, review and analyze historical records of CBEP pathogens; to enter these data into an EIDSS-linked database that would allow rapid identification of unusual patterns of clustering of disease anywhere in the country.

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Revision History

Version #	***Date***	***Description***
1	*07/03/11*	*Pre-proposal from 2 different sources*
2	*07/28/11*	*Pre-proposal combination efforts with team*
3	*08/5/11*	*Combined pre-proposal with brainstorming*
4	*08/17/11*	*Form A outline and collaborators fully chosen*

5	*09/06/11*	*Form A final specific aims completed with collaborators*
6	*09/30/11*	*Clinical studies outlined*
7	*10/06/11*	*Expansion of clinical proposal and confirmation of new field studies needed*
8	*10/13/11*	*Expansion of molecular work and phage and proteomic interest added as outlines*
9	*10/14/11*	*Expansion of clinical work new version*
10	*10/16/11*	*Detailed experimental plan complete*
11	*10/17/11*	*Collaborator updates on background text*
12	*10/30/11*	*Text changes and cleaning up background and introduction, specific aims re-written, DTRA required sections outlined, final revision from NCDC*
13	*11/02/11*	*Eliava additions*
14	*11/30/11*	*LSRC additions and revisions*
15	*12/14/11*	*Completed clinical study and expansion of text on clinical studies*
16	*01/05/12*	*Clean up text and collaborators content revised*
17	*01/13/12*	*Modifications to final Form A draft and collaborator review (institutes)*
18	*01/22/12*	*Text changes and modifications to match Form B from mapping project to Form B*
19	*01/25/12*	*Edits from Eliava, LSRC, and NCDC*
20	*01/29/12*	*Final scrubbing of document*
21	*01/30/12*	*Final formatting, grammar check, and CH2MHill edits, updating collaborators*
22	*2/22/12*	*Updated collaborators and budget*
23	*05/06/12*	*Removal of mouse study, update to the budget, and updates of collaborating team and management team*
24	*5/28/12*	*Edits to specific aims and rational/approach based research rewrite per DTRA direction.*