

TADR ACTIVITY PROJECT (TAP)

Georgia 712012

Project Proposal

Latest Revision: 02 May, 2014

Project Title

NCDC Lugar Center Regional Integration: Kafkas University Partnering for Molecular Epidemiology of *B. anthracis* and *Brucella* species in Turkey

Project Summary

Infectious microorganisms do not respect man-made borders, and this is certainly true of livestock-borne zoonotic diseases. It is therefore vital that effective collaborative links are built between the NCDC Lugar Center and institutions in neighboring countries to address shared common bacterial threats. One such institution is Kafkas University, which is located in the city of Kars in northeastern Turkey. The university's veterinary school serves a primarily agricultural region adjacent to the Georgian/Turkish border where diseases including anthrax and brucellosis are endemic. Molecular typing tools are available to the group at Kafkas but these are not as sophisticated as those currently available at NCDC Lugar Center. Thus a study of prevalent strains on both sides of the border, using the same high resolution molecular tools, would enable researchers to build a comprehensive picture of the regional epidemiology of these important pathogens. To achieve this aim, the team at NCDC Lugar Center will provide training to their Turkish colleagues in how to employ the state of the art capabilities currently available at NCDC Lugar Center. The expectation is that this project will serve as a foundation and catalyst for future collaborative efforts between Georgian and Turkish researchers.

Institutes

Collaborating Institute

Name: National Center for Disease Control (NCDC) Richard Lugar Center for Public Health Research (NCDC Lugar Center)

Street Address: 9, M. Asatiani Str.

City: Tbilisi

Region: Caucasus

Country: Georgia

Zip Code: 0186

Name of Signature Authority: Amiran Gamkrelidze

Tel: +995 32 2239 89 46

Fax: +995 32 2243 30 59

Email: a.gamkrelidze@ncdc.ge

Collaborating Institute

Name: Kafkas University

Street Address: 36100 Campus University

City: Kars

Region: Kafkas Paşaçayırı

Country: Turkey

Zip Code: N/A

Name of Signature Authority: Mitat Sahin

Tel: +90 4742128821

Fax: +90 4742126829

Email: mitats@hotmail.com

Collaborators

Name: Jason Farlow, PhD Title: Collaborator
Institute: AEP
Address: NCDC Lugar Center, 14 Kakheti Highway Tbilisi 0152 Georgia
Tel: 995 32 224-3427 Fax: N/A E-mail: farlow@hotmail.com

Name: Mikeljon Nikolich, Ph.D. Title : Collaborator
Institute: WRAIR and USAMRU-G
Address: 503 Robert Grant, Silver Spring, MD 20910
Tel: +1 301031909469 Fax: N/A E-mail: Mikeljon.p.nikolich.civ@mail.mil

Name: Les Baillie, Ph.D. Title: Collaborator
Institute: Cardiff University School of Pharmacy and Pharmaceutical Sciences
Address: Cardiff CF10 3BB
Tel: +44 0290208075535 Fax: +44 02920874149 E-mail: baillieL@cf.ac.uk

Name: Hugh Dyson Title: Collaborator
Institute: Defense Science and Technology Laboratory
Address: Porton Down, Wiltshire SP4 0JQ, UK
Tel: +44 (0) 7884 002181 Fax: +44 (0) 1980 613345 E-mail: ehdyson@dstl.gov.uk

DTRA Representatives

Name: Gavin Braunstein, Ph. D. Title: Science Manager
Address: 8725 John K. Kingman Drive, MSC 6201, Ft. Belvoir, VA, USA, 22060-6201
Tel: +1 703-767-4862 Fax: +1 703-526-7794 Email: gavin.braunstein@dtra.mil

Name: David Hall, CIV Title: BTRIC GG COR
Address: 8725 John K. Kingman Drive, MSC 6201, Ft. Belvoir, VA, USA, 22060-6201
Tel: +1 703 767-7791 Fax: Email: David.Hall@dtra.mil

Integrating Contractor Representatives

Name: Richard J Obiso Jr., PhD Title: CBR Manager
Address: 4071 Childress Road, Christiansburg, VA 24073
Tel: +1-540-831-9234 **Fax:** Email: rik@attimogroup.com

Name: Magda Metreveli, MD, PhD Title: Deputy CBR Manager
Address: 4 Freedom Square, GMT Plaza, 0105 Tbilisi, Georgia
Tel: +995 595 36420 **Fax:** +995-322-92-29 Email: Magda.Metreveli@ch2m.com

Detailed Project Information

I. Project Description (Introduction and Overview)

Bacillus anthracis, the etiologic agent of anthrax, is a monomorphic member of a highly diverse group of endospore-forming bacteria. The genus *Bacillus* contains at least 51 described species and many other species of uncertain taxonomic status (1). *B. anthracis* spores are typically found in soil, from which they may spread via contaminated dust, water, and materials of plant and animal origin. The toxins produced by the vegetative bacterium are associated with its virulence and they differ from the toxins produced by other *Bacillus* species. Although anthrax is primarily a disease of herbivores, humans may contract anthrax directly or indirectly from animals (2). Three forms of human anthrax have been described. The most common one, cutaneous anthrax (which accounts for 95-99% of human cases worldwide), usually results from handling contaminated animal products. Infection occurs through a break in the skin and the lesions generally occur on exposed regions of the body. After an incubation period of 2-3 days, a small papule appears, a ring of vesicles develops around the papule during the next 24 h, and the papule subsequently ulcerates, dries and blackens to form a distinctive eschar (dead tissue). Less than 20% of untreated cases of cutaneous anthrax are fatal. In fatal cases, generalized symptoms may be mild (malaise and a slight fever) or absent prior to a sudden onset of acute illness characterized by dyspnea, cyanosis, severe pyrexia, and disorientation followed by circulatory failure, shock, coma and death, all within a few hours (3). Concomitant with the severe signs of illness, the number of anthrax bacilli in the blood increases rapidly and reaches a maximum concentration, during the last few hours of life, of 10^7 - 10^9 CFU/ml. Two very severe, but much less common forms of human anthrax are gastrointestinal anthrax caused by the consumption of contaminated animal products, and pulmonary anthrax caused by the inhalation of *B. anthracis* spores in contaminated animal carcasses and wool. Those two forms of anthrax are much more severe than cutaneous anthrax because they are more likely to result in the rapid dissemination of bacteria to regional lymph nodes and fatal septicemia.

B. anthracis is extremely difficult to eradicate from endemic areas because of the environmental stability of its spores, which remain viable in soil for many years, and because bacterial persistence does not depend on animal reservoirs (4). Despite the development of anthrax vaccines for animals and humans, the disease continues to be endemic in many countries, particularly those lacking efficient vaccination policies. Relatively few studies have been completed characterizing the strains of *B. anthracis* present in Turkey (5, 6). In view of a significant increase in the number of anthrax cases in regions near Turkey, such as Georgia, in recent years, this situation is of great concern.

Possessing an in-depth understanding of the genetic composition of the Turkish *B. anthracis* isolate population will be critical for the timely identification of strains and the ability to trace them to their origin. Furthermore, elucidating differences in the antigenic composition of Turkish strains will be invaluable for improving vaccine intervention strategies to curtail *B. anthracis* outbreaks, either naturally occurring or intentionally caused as the result of a bioterrorism attack. Additionally, advancing our understanding of the association between specific genes and the etiologic agent's high-virulence phenotype may help to improve strategies for anthrax control and prevention, and eventually enable the disease to be eradicated from endemic areas.

Brucellosis is the most common bacterial zoonosis, resulting in significant economic losses globally due to reduction in animal populations, as well as debilitating human disease. Although the picture of global brucellosis morbidity is not completely clear, brucellosis is found on almost every continent, and at least 500,000 people are infected annually (7). *Brucella melitensis* is the most frequent cause of human

brucellosis accounting for an estimated 96% of cases worldwide. Human infection occurs from exposure to infected animal abortion products or contaminated dairy products, and it is accepted that control of the human disease can only occur through control and eradication of the disease in animals.

Brucellosis is currently under-diagnosed and under-reported in Turkey (8) and worldwide due to nonspecific clinical manifestations, the limitations of current diagnostic tests, and the widespread use of non-prescription antibiotics in many endemic countries. Brucellosis infection in humans can be challenging to diagnose as its presentation is nondescript and mimics many other conditions (9). In the early presentation (days to weeks after exposure), non-specific symptoms such as fever, fatigue/malaise, myalgia and arthralgia predominate. Some patients proceed to develop focal complications, with osteoarticular complications being somewhat common and very debilitating (9). Other patients may have chronic, mild symptoms for many months, making brucellosis difficult to recognize as the cause of their condition. These patients have traditionally been classified as having chronic brucellosis, but the utility of this classification is not established. Additionally, a certain percentage of patients relapse, usually with non-specific symptoms, even after an extensive course of initial therapy, however, the contribution of lack of treatment compliance and re-infection of these patients to relapse is not known.

Human brucellosis is caused by five classical *Brucella* species (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*), of which *B. melitensis* is considered the most virulent for humans (10). *B. melitensis* is highly infectious for humans with an ID₅₀ of 10 cells per person, and it is virulent in other mammal hosts such as bovine cattle, though sheep and goats are its preferred host (1). *B. melitensis* infections are especially problematic as the most commonly used attenuated *B. abortus* cattle vaccine (Strain 19) fails to protect from disease caused by *B. melitensis* (11). Human cases of brucellosis are acquired through contact with infected animals and through consumption of contaminated dairy products. Adherence to traditional farming practices and the consumption of unpasteurized dairy products contribute to the high incidence of brucellosis in Turkey (8).

The *B. abortus* RB51 vaccine has also been used successfully in selected countries to aid in the control of bovine brucellosis (caused by *B. abortus*). A complicating factor of vaccination is that classical serologic diagnostic protocols cannot distinguish animals vaccinated with Strain 19 from infected animals and diagnosis during latent infection can be particularly challenging. Only the use of the rough *B. abortus* vaccine RB51 in bovine cattle addresses this issue because it lacks the dominant antigen, LPS O-sidechain, but this vaccine is yet to be accepted worldwide. Meanwhile the utility of vaccination of livestock with Strain 19 is significantly decreased by the inability to distinguish between vaccinated animals and those animals with active infections without sophisticated laboratory assays. These issues underscore the need for improved detection and diagnostic capabilities in the primary livestock hosts of *Brucella*. This project will help address this issue using high resolution genetic characterization techniques.

Several studies previously carried out in Turkey and Georgia have described genetic relationships among *B. anthracis* circulating in the region. In 2006, MLVA 8 genetic subtyping by Merbiashvili et al on 18 field strains and five FSU vaccine strains employed for livestock throughout the FSU revealed that within Georgia, Caucasian *B. anthracis* strains fell within the Keim et al previously defined A.3.a subgroup in two genotype clades shared with regional Turkish isolates. Similarly, Durmaz et al studied 251 *B. anthracis* isolates from human (93 isolates), animal (155 isolates), and environmental (three isolates) sources throughout Turkey and revealed a total of 12 distinct MLVA 25 A3.a subtypes. Ortatatli et al also revealed genetic diversity patterns among 55 *B. anthracis* isolates from 16 distinct regions of

Turkey. These authors identified three geographically related subgroups within three distinct regions and revealed genotype dispersal patterns consistent with potential trans-boundary contamination from livestock.

Data presented at the 2012 Munich Medical Biodefense Conference by Khmaladze et al based on whole-genome derived SNPs (and MLVA) showed two distinct genetic populations of *B. anthracis* exist in Georgia with one sharing conserved genetic signatures with neighboring Turkish strains (manuscript in preparation).

Studies to examine the genetic diversity and regional distribution of *Brucella* species in Georgia found high genetic diversity between isolates, and geographic distribution was dependent on genotype. A study of 35 isolates of human and animal *Brucella* obtained in Georgia in 2009 and 2010 using MLVA-8 analysis illustrated the high diversity between isolates (Jgenti *et al.* 2013). It was found that only 2-3 strains were located in one genotype cluster, with some genotypes showing restricted geographical distribution, and others were disseminated among different regions of Georgia. One genotype cluster contained animal and human isolates from the same region, revealing a potential source of human infection.

A study similar study was conducted in Turkey with 162 human *Brucella* isolates collected from 2001-2008 and analyzed using MVLA-16Orsay (Kilic *et al.* 2011). A total of 105 genotypes were recorded, with 73 being represented by a unique isolate, and 32 including 2-8 isolates. As was found in Georgia, a number of the isolates had restricted geographic distribution. In addition, spanning tree analysis of published *B. melitensis* isolates, using MLVA-11Orsay data, indicted that Turkish isolates were most closely related to the neighboring countries' isolates included in the East Mediterranean group.

A regional synthesis of data and expertise between Georgia and Turkey with regard to the molecular epidemiology of these two important trans-boundary zoonotic diseases is needed. The current TAP provides the opportunity to advance this goal and begin integrating epi-surveillance data and advanced molecular sub-typing techniques for both pathogens between Turkey and Georgia. Furthermore, this effort supports more enhanced disease control strategies through providing subtype-specific distributions of endemic and potential newly emerging *B. anthracis* and *Brucella* variants. This collaboration will provide: 1) shared epi-surveillance information on select project strains between Turkey and Georgia pertinent to regional risk assessments for anthrax and brucellosis, 2) deeper insight into the tracing of mobile pathogen lineages for each pathogen and their originating environmental foci threatening human and animal health across the region, and 3) collaborative training in advanced molecular techniques that will enhance standardized interrogation of *B. anthracis* and *Brucella* isolates by Georgian and Turkish scientists while providing the opportunity for continued regional collaborations in public health research.

II. Scientific Goals

This project is designed to leverage the scientific expertise and capabilities vested in the NCDC Lugar Center to address an international public health issue, provide training in molecular biology techniques to Turkish researchers at Kafkas University, improve the understanding of the molecular epidemiology of bacterial zoonotic pathogens which threaten the health of Georgian and Turkish citizens, and establish an active collaboration between Georgian and Turkish researchers that will provide a foundation for future joint research efforts. This collaborative project will involve NCDC Lugar Center, Kafkas University, WRAIR and Professor Les Baillie (CU). Professor Baillie's role will be to act as a facilitator as

he is well known to all of the study participants and has expertise in this area. Dr. Adam Kotorashvili (NCDC Lugar Center) will be the Georgian lead for the project in conjunction with Dr. Mikeljon Nikolich (WRAIR, USAMRU-G), and Dr. Nino Trapaidze (WRAIR-USAMRIID Clinical Research Unit, USAMRU-G). Dr. Kotorashvili, with two Georgian researchers, and Dr. Nikolich will visit Kafkas University for finalization of isolate selection, sample processing, DNA extraction, and sterility testing. Following receipt of sterile DNA samples, NCDC Lugar Center will host Prof. Mitat Şahin and 2 staff from the Şahin laboratory to review the molecular diagnostic methodologies employed by WRAIR and NCDC.

This project will focus on the following objectives:

1. Bacterial DNA extraction from species of interest
2. Molecular analysis of *B. anthracis* isolates
3. Molecular analysis of *Brucella* isolates

III. Technical Approach and Methodology

The duration of this project will be one year. The goal of the project is to characterize the genetic diversity displayed by Turkish strains of *B. anthracis* and *Brucella spp.* specifically for integration into a comparative regional data set. This will provide the opportunity to elucidate genetic patterns between Turkish and Georgian strains relevant to the molecular evolution, ecology, and epidemiological tracing of these pathogens.

The project will comprise three main technical elements: bacterial DNA extraction, molecular analysis of *B. anthracis*, and molecular analysis of *Brucella* species. All bacteriological testing will be performed under biosafety level (BSL) -2 conditions using BSL-3 practices, where appropriate, in the BSL-3 laboratory (BSL-3 trained personnel only), and will be confirmed using control ATCC strains.

Training: All Turkish personnel will be trained by WRAIR and NCDC Lugar Center collaborators. Training will take place at the Lugar Center, Tbilisi, Georgia, using control ATCC strains where appropriate. Training will encompass every aspect related to the activities described in this proposal, including:

- Sample preparation, including purification and storage
- DNA extraction techniques, including verification of sterile samples
- Template DNA preparation
- Molecular subtyping techniques for both pathogens, including SNP and MLVA analyses
- Training in DNA sequencing techniques and bioinformatics analysis
- Data analysis

Currently, the group at Kars isolates pathogens from environmental samples using basic microbiological techniques. Strains are then sent to Ankara where they are typed and the results are returned to Kars. Therefore, this group would benefit from training in sample processing, molecular genetic analysis and bioinformatics techniques. Activities will begin when Georgian collaborators travel to Turkey to finalize strain selection. Project participants from Kafkas University will provide DNA samples from the strains selected and transport them, either by shipping or hand-carry, to the Lugar Center. Training will then continue at NCDC Lugar Center during which time the molecular genetic analysis will take place. Training on the ABI 3130xl and Illumina MiSeq sequencing platforms from sample preparation to data analysis will be provided. Library preparation, and genome sequence annotation, analysis, and assembly will be addressed.

Data Analysis

While the ability of Turkish researchers to gain experience in state of the art molecular typing methods is a welcome outcome of the project, the principal aim is to generate data that will enable professionals on both sides of the Georgian-Turkish border to gain a clear picture of how these pathogens are related. Pathogens, unlike humans, do not recognize borders and so it is vital for disease control to determine whether particular strains are common to Georgia and Turkey and indeed the rest of the Caucasus. Thus molecular typing techniques for both pathogens that have already been employed in Georgia in various collaborative efforts will be applied to the Turkish isolates.

The bioinformatics analysis of molecular typing data is a particular skill and we are fortunate that amongst the collaborators on this project we have individuals who are skilled in the analysis of these types of data. While their input will be a key to the success of the project, the overall goal is empower the Georgian and Turkish researchers to analyze the data themselves thus enhancing their own in-house capabilities.

Training for Turkish researchers at NCDC Lugar Center

1. Bacterial DNA extraction, purification and storage
 - a. Bacterial culture and inactivation
 - b. DNA extraction and purification
2. Sequencing on Sanger AB 3130xl platform (Applied Biosystems)
 - a. Introducing to the sequencing platform
 - b. Preparation of PCR reaction for DNA sequencing
 - c. BigDye reaction setup
 - d. Set up program and run instrument
 - e. Introduction of computer software for genome sequence annotation and analysis
3. Sequencing on Next Generation Sequencing (NGS) Illumina MiSeq platform
 - a. Introduction to sequencing platform
 - b. Genomic DNA QC
 - c. Evaluate quality and quantity of genomic DNA samples. Calculate DNA concentration with Qubit, sample preparation for gel electrophoresis.
 - d. Fragment DNA using Covaris and run sample on bioanalyzer to verify the size of fragmented DNA using a DNA 1000 Chip
 - e. Library preparation
 - f. Library Validation – Qubit, Bioanalyzer
 - g. Set up a run on the Illumina MiSeq
 - h. Template DNA preparation
 - i. Reagents preparation
 - j. Loading sample, making sample sheet , run MiSeq
 - k. Introduction to Different Bioinformatics tools
 - l. CLC bio, Velvet, Casava, Mauve
 - m. Sequence QA/QC, map to reference, de novo assembly

Objective 1: Bacterial DNA extraction

Sterile DNA samples from strains selected from the library at Kafkas University will be either packaged using standard packaging and shipped to the NCDC Lugar Center, or hand-carried by project participants. Samples will be used for genetic analysis performed through collaboration between NCDC Lugar Center staff, Kafkas University, and UK collaborators. Bacterial DNA will be extracted from selected Georgian strains using SOPs provided by NCDC. Bacterial culture, DNA extraction and purification conducted by NCDC Lugar Center staff will be conducted in the Lugar Center BSL-3 facility. A summary of the process is described below.

DNA preparation

In order to complete the molecular characterization of isolates, a substantial amount of genomic DNA will be required. Pure culture isolates of suspected *Brucella* species will be plated on appropriate media for five days. Pure culture isolates of suspected *B. anthracis* will be cultured on blood agar for 24 hours. Several loops of suspected cultures will be placed in 1.5 mL microcentrifuge tubes. *Bacillus anthracis* will be heat killed first, and then both *Brucella* samples and *B. anthracis* samples will be incubated for one hour in 50% v/v chloroform. Sterility of samples will be determined by removing 1-10% of the final volume and incubating in appropriate growth media. At day +3 an aliquot will be streaked onto appropriate agar and incubated ON, and sterility culture re-incubated. At day +7 the streaking procedure will be repeated. If no growth is observed at either time point then the preparation can be considered sterile. Primary and secondary containers will be decontaminated for 30 minutes with 1% NaOCl, and stored at -20°C. After sterility confirmation and surface decontamination, samples will require BSL-1 containment. Once sterility has been confirmed, genomic DNA will be extracted using a commercially available extraction kit following the manufacturer's instructions for bacterial cultures. Purified DNA will be aliquoted and frozen for future analysis.

Objective 2: Molecular analysis of *B. anthracis*

Molecular analysis will include: a) use of real-time PCR assays with Single Nucleotide Repeats (SNPs) to determine major genetic groupings, and b) use of Variable Number Tandem Repeat (VNTR) analysis to provide higher resolution genetic differentiation in a Multiple Locus VNTR Analysis (MLVA). Georgian reference strains from the collection at NCDC will be used for comparison.

SNP Analysis

Single nucleotide polymorphism analysis of *B. anthracis* has been established as an extremely valuable tool for molecular characterization of this species (12). A SNP tying panel has previously been established at NCDC based on canonical and Georgia-specific SNPs. This type of analysis will be useful for the characterization of Turkish isolates in addition to the comparison of Turkish and Georgian isolates. This will be particularly informative when comparing isolates originating near the border between Georgia and Turkey.

VNTR Analysis

Multiple locus VNTR analysis is used to characterize highly monomorphic pathogens such as *B. anthracis* and *Brucella* species (13). A MLVA-25 technique will be utilized to analyze *B. anthracis* isolates. These techniques have recently been adapted for use on the ABI 3130xl platform available at the NCDC Lugar Center in a WRAIR-NCDC DTRA-funded strain characterization collaboration. This high-throughput technique allows many strains to be analyzed with relatively few reactions.

Objective 3: Molecular analysis of *Brucella* species

Currently, identification of *Brucella* isolates relies on a set of traditional microbiological tests that are long and tedious (requiring up to 21 days for completion). Techniques employing PCR that can rapidly identify *Brucella* and differentiate between species, biotypes and strains within the genus have been implemented in clinical laboratories with various degrees of success. PCR applications have proved to be more sensitive than serological tests for the detection of brucellosis and PCR allows the earliest detection of *Brucella*, starting about ten days after infection.

Molecular characterization of *Brucella* species will include: a) genetic determination of species using the Bruce Ladder conventional PCR assay (15), b) use of real-time PCR Single Nucleotide Repeat (SNP) assays to determine major species/biotype groupings, and c) use of VNTR analysis to provide higher resolution genetic differentiation and inference of clonal associations. The SNP assays will be selected to differentiate the *Brucella* species and other major genetic groupings within the genus. For the highest resolution subtyping, a MLVA approach will be employed. Species-specific SNP analysis, MLVA-7, and MLVA-15 techniques were established for *Brucella* species by NCDC with WRAIR collaboration during the DTRA funded CBR GG-17 project. Georgian reference strains will be used for comparison in each technique. Reference strains are maintained at the NCDC repository for this purpose. Molecular biology techniques circumvent problems associated with classical biotyping protocols by using validated markers and unambiguous identification of groups.

Bruce Ladder

This multiplex PCR assay is a robust method for differentiating between different *Brucella* biovars (16). This assay is safe, fast, requires minimal sample preparation, and can successfully identify all known *Brucella* species in a single test-tube format. This assay was recently established at NCDC in the WRAIR-NCDC DTRA-funded strain characterization collaboration and can be applied in this project.

Real-time SNP Analysis

Real-time SNP analysis assays have been optimized to rapidly identify *Brucella* isolates (17). This type of assay allows for rapid, unambiguous identification of *Brucella* isolates to the species level.

VNTR Analysis

The proposed MLVA assay can be easily applied to large numbers of *Brucella* isolates, either manually or in an automated fashion, and will complement the other biotyping methods included in this proposal. As with other DNA-based assays, MLVA utilizes non-infectious material, and the resulting data can be compiled in a database to be used in further characterization studies (18).

VNTR PCR amplicons will be discriminated through electrophoretic analysis with a CEQ 8000 or an ABI PRISM 3130xl automated fluorescent capillary DNA sequencer (both available at the NCDC Lugar Center), though we plan to use the ABI system for this project. Fragment analysis for the ABI system will be performed with the GeneScan and GeneMapper software packages (Applied Biosystems, for 3130xl). Fragment sizing will be accomplished by comparison to a custom-made LIZ-dye-labeled size standard and custom macro programs set up in GeneMapper will allow automated scoring of VNTR alleles. MLVA will be performed on this sample set in duplicate to examine the reproducibility of the analysis.

How the MLVA is applied to the collection of *Brucella* DNA samples from Kafkas University will depend upon the number of samples selected by the collaborators to be analyzed within the reasonable scope of this project. At minimum, 20 isolates will be used for all the samples and more exhaustive MLVA-15

(NAU marker system, Hyun et al) will be completed for as many Kafkas University strains as is possible within this TAP.

Throughout experimentation and data analysis, DNA samples remain the property of the originating lab. Subsequent studies using these samples will require additional approval, and remaining DNA samples should be destroyed upon completion of the proposed studies.

IV. Expected Results

The successful implementation of this project will:

- Leverage the scientific expertise and capabilities vested in NCDC Lugar Center to address an international public health issue.
- Contribute to improved understanding of the molecular epidemiology of bacterial pathogens which threaten the health of Georgian and Turkish citizens.
- Facilitate an active collaboration between Georgian and Turkish researchers that will seed future joint research efforts and engage regional comments.

V. Tasks

Task Description			
Project management, reporting, and administration			
	Description of Deliverables	Responsible Party	Completion Schedule
Setup	Procurement of materials and project management	BTRIC GG	Q1
Training	Collaborators from Georgia travel to Kars; SOP development	NCDC/Collaborators	Q1
	Turkish scientists travel to Georgia	NCDC/Collaborators	Q2
Molecular Analysis	SNP, VNTR analysis	NCDC/Collaborators	Q2-Q3
Final write-up and final report	Final report, manuscript preparation	NCDC/Collaborators	Q4

VI. Project Management

This project will be managed as a collaborative effort between NCDC Lugar Center, Kafkas University, WRAIR and Professor Les Baillie (CU). The project manager will communicate through BTRIC GG with US Collaborators via e-mail or conference calls and will be responsible for preparation of monthly reports and project presentations for international conferences. The project manager will also be responsible for financial management. Duties of the project coordinator are detailed below.

Project reporting

- Consolidation of monthly reports from participating institutes (assemble and quality check deliverables);
- Preparation of a short executive summary for the monthly report;
- Leadership in preparation of project presentations, abstracts for conferences and international meetings;

- Publications in peer review journals;
- Regular communication with the US collaborators;
- Participation in monthly project calls;
- Organization of the meetings of the TAP project participants (all subprojects) to discuss the project progress;
- Participation in international conferences and meetings.

Team member responsibilities:

- Timely and quality completion of assigned project activities;
- Familiarity with the science work plan and project SOPs and protocols;
- Record the time spent on the project on a daily basis;
- Record the project work in a laboratory book/journal;
- Technical competence in the area of their responsibility;
- Matrixed skill set - overlapping skills between team members allow for changes in assignments at a later date;
- Participation in international conferences.

VII. Meeting Goals and Objectives of CBR Program

The project will contribute to improving animal and human health in Georgia and Turkey. It will enhance Georgian scientific connectivity with regional partners such as Turkey and will provide a model for future networking opportunities with other countries in the region.

This project addresses the following CBEP cooperative research priorities:

- Establish genomics and proteomics capabilities for identification and comparison of BTRP priority pathogens.
- Expand the diagnostic and research training of a new cohort of investigators and include new disciplines such as computational biology and bioinformatics.

VIII. Pathogens

	Biosafety Level (Diagnostic Quantities)			
Pathogen	1	2	3	4
<i>B. anthracis</i>			X	
<i>Brucella spp.</i>			X	

Please note that microorganisms will only be cultured to isolate DNA. No other culture or characterization is authorized under this project.

IX. Approximate Budget

See Form B

X. References

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