Project title: Risk of Emerging Infections from Insectivorous Bats in Ukraine and Georgia

Introduction and review

Epidemics of emerging infectious diseases are on the rise. The novel coronavirus strain SARS-CoV-2 (preliminary originated from bat) has resulted in pandemic, the biggest quarantine in human history, the global interruption of all traffic, the international political instability, and the variation in the global economy. Clearly, predicting disease emergence is of critical interest but capacities to anticipate where and when diseases will emerge are limited.

Bats are the hosts of a wide range of emerging zoonotic viral and bacterial pathogens. Bats have unique biology and may play a role in maintenance and transmission of infectious agents to other vertebrates. The significance of bats harboring emerging pathogens that may potentially affect humans in Ukraine and Georgia has been scarcely investigated to date. The project's objectives are to assess the role of bats as a natural reservoir for pathogens of relevance to human and animal health in Ukraine and Georgia, to investigate factors could influence the assemblage of pathogens in bat populations and how these overall changes can drive to disease emergence in humans and domestic animals; to build capacity to create a sustainable surveillance system (active studies of bats in Ukraine haven't been conducted) that may help to detect, prevent and predict disease emergence in the region. The project will focus to detect and determine the geographic range of viral (coronaviruses, filoviruses, paramyxoviruses, orthomyxoviruses, lyssaviruses), and bacterial (Brucella spp, Leptospira spp, Yersinia spp) agents circulating in bat populations, as well as determine their evolutionary relationships with pathogens of known relevance for human and animal health and its linkage with different environmental factors. These studies will not only allow the identification of pathogens, assessing the role of bats as the source of zoonotic diseases in Ukraine and Georgia, but also will contribute significantly in the improvement of disease surveillance systems in wildlife.

This project has the potential to advance our understanding on how species assemblages modify host-parasite interactions and how urbanization influences the dilution or amplification effects between biodiversity change and disease emergence as well as data driven risk assessment; Expected findings are of interest for the fields of ecology and evolution of infectious bacterial and viral diseases, early warning systems, and global health; This project is expected to generate data to elucidate the efficacy (or lack thereof) of biodiversity conservation at the local level (e.g., around human settlements) to reduce the burden of infectious diseases.

Creation of a research network for the surveillance and early detection of known and potential high-consequence pathogens for humans and domestic animals in East Europe; Advance Georgia and Ukraine capacity to assess virulence of viral agents found in bats by assessing the evolutionary relationships of novel virus with known high-consequence pathogens; Develop ecological models for the prediction of high-consequence pathogens in unexplored regions of Georgia and Ukraine and neighboring countries based on landscape configuration.

Results obtained will also contribute to the development and implementation of emergency response and preparedness plans in a future. Capacity building will focus on training local scientists in safe and effective techniques for bat capture, sampling, and biosafety measure in the field and in the laboratory. Our project will establish a self-sustainable platform in the both countries for basic pathogen discovery in wildlife using modern laboratory screening technologies while complying with international biosafety requirements.

What is the goal of the project?

The main objectives for this project are: Detect high consequence viral (coronaviruses, filoviruses, paramyxoviruses, lyssaviruses and bunyaviruses, orthomixoviruses) and bacterial (*Brucella spp, Leptospira spp and Yersinia spp*) agents in bat populations in Ukraine and Georgia; Investigate how landscape biodiversity change (pristine, rural, urbanized settings) could influence the assemblage of high consequence viral and bacterial agents in bat populations and how these overall changes can drive to disease emergence in humans and domestic animals. Tracing the evolutionary relationships between bat borne agents and their known close pathogenic relatives causing disease in humans and animals are also central for this project*;* Build a sustainable harmonized surveillance network for the early detection, full genomic characterization, data storage and analysis of high consequence viral and bacterial agents associated with bat populations in Ukraine and Georgia, with a long-term vision to expand this working.

What is the problem?

Biodiversity is essential for ecosystem functioning; the globally accelerated biodiversity loss due to urbanization and agriculture will likely create unexpected species assemblages and interactions at different biodiversity scales (macro and microbiota) (Johnson, 2017). Emerging infectious diseases have been linked with biodiversity changes, where most recent epidemics have had a confirmed wildlife origin (Johnson CN, 2017). Nevertheless, the exact point where biodiversity change drive to the emergence of pathogens that affect humans and animals remains unclear (Rohr 2020). The biodiversity-disease relationship has been under intense scrutiny in the field of ecology and evolution during the last decade (Rohr 2020, Randolph 2012, Wood 2013, Lafferty 2013, Wood 2013).

Overwhelming evidence links the emergence of high-consequence pathogens with bat communities and human settlements. Examples include Filoviruses (e.g., Marburg, Ebola viruses) (Leroy 2005, Olival 2014, Towner 2009, 2007, Yang 2017), Lyssaviruses (e.g., rabies, European Bat Lyssavirus 2) (Arai 2003, Aznar-Lopez 2013, Harris 2006, Kuzmin 2006), Paramyxoviruses (e.g., Nipah, Hendra) (Baker 2013, Chua 2002), Coronaviruses (e.g., SARS-CoV, SARS-CoV-2, MERS) (Annan 2013, Li 2005, Memish 2013), Bunyaviruses (CCHF-like viruses) (Müller 2016), and Orthomyxoviruses (2 new subtypes: H17N10 and H18N11) (Tong, 2013). Strikingly, there is limited comprehensive knowledge of the evolutionary and ecological relationships of these pathogens with the composition of bat species or the levels of habitat degradation.

Additionally, available information is biased towards data generated during epidemics, neglecting the understanding of pathogen circulation and characterization before outbreaks. This reactive instead of preventive approach prevents the understanding of the factors that modify pathogen circulation in the original wildlife reservoir.

The striking abundance and diversity of bat coronaviruses and their close similarity to those found in pandemic respiratory syndromes affecting humans and animals worldwide, corroborate the critical role bats may play as the origin for the global dissemination of high consequence infections. Likewise, more evidence accounting for the extraordinary taxonomical breadth of bat viruses reveals, at least, 248 novel viruses belonging to 24 virus families identified in different parts of the world during the period of 1991-2016 (Young 2016, Mühldorfer 2011). In addition, several bat-borne high-consequence pathogens found across Europe, Africa, and East Asia also unveil long evolutionary relationships perhaps associated with long-term global disseminations. As an additional example of the global spread of bat viruses, relatives of filoviruses originally thought to be circumscribed to Africa have been discovered in bats in China (Yang 2017). Paramyxovirus, Orthomyxovirus, and Bunyavirus have been also found in bats from different countries around world, suggesting unnoticed and widespread risk of emerging diseases to humans (Conrardy 2014, Müller 2016).

What other people do?

Rhinolophidae and Vespertilionidae bat families have been most frequently found infected with high-consequence pathogens and their relatives (Ostfeld 2017, Civitello 2015, Yang 2017). Western Europe is a nucleus of diversity for these bat families. For example, there are between 28 and 30 species of Rhinolophidae and Vespertilionidae bats in Georgia and Ukraine alone, both countries sharing around 80% of the total number of bat species reported (Zagorodniuk 2017, Gorlov 2016). Our recent studies in Georgia revealed circulation of a large diversity of bat-borne pathogens (Bai, 2017), including a striking diversity of SARS- and MERS-like coronaviruses [Urushadze L, Velasco-Villa A. et al. in prep.]. In the same region, Ukraine reported stable bat-borne pathogen circulation (Klueva 1991, Selimov 1991, Sonntag 2009), and detection of novel viruses, potentially zoonotic, from the Circoviridae family (genus Cyclovirus) [Kemenesi, 2017]. The location of Georgia and Ukraine between Europe and Asia, and the outstanding dispersal of bats (800-1600 km, linked to migration and dispersal after perturbation), make these countries a natural corridor for pathogen exchange and an ideal region to study host-parasite evolution and the biodiversity-disease relationship using high-consequence pathogens in bats as model system.

You are going to do?

Scientific project tasks: Assess the taxonomical diversity of potentially endemic viral (coronaviruses, filoviruses, paramyxoviruses, lyssaviruses and bunyaviruses, orthomixoviruses) and bacterial (*Brucella spp, Leptospira spp and Yersinia spp)* agents associated with bats living in pristine and urban settings in Ukraine and Georgia (Year 1-3); Investigate the evolutionary relationships between these agents and those known to cause disease in humans and domestic animals using comparative genomics approaches (Year 1-3); Monitor potential seasonal variations in positivity rates, relative composition or overall diversity shifts for bats and their associated bacterial and viral agents (Year 1-3); Assess potential associations between environmental variables and the bacterial/viral agent diversity in Georgia and Ukraine to model risk and disease emergence (or cryptic circulation), by using ecological niche modeling approaches (Year 1-3); Determine the linkages between landscape structure and bat species diversity (Year 2-3); Identify the effect of bat community composition on the occurrence of high-consequence pathogens. (Year 2-3).

Non-scientific project tasks: Foster a sustainable exchange of technology and scientific expertise among institutions from all participating countries to create a solid regional research network; Nurture a culture of biosecurity and biosecurity to improve field and laboratory work with high consequence agents in the region; Improve local capacity for the investigation, early detection of high consequence viral and bacterial agents based on high throughput harmonized standard operating procedures from CDC; Creation a self-sustainable passive surveillance disease network in sick or dead bats across Ukraine and Georgia to complement field studies; Develop local capabilities for the storage, management and analysis of complex genomic data, data interpretation.

What is new?

New information about present or absent of EDPs in bat in Ukraine and Georgia; Standardization and regulation of research methods for EDPs in Ukraine; Spatial representation of the distribution of the studied infections and their causative agents in the defined areas in Ukraine and Georgia; Presentation and publishing new results; State authorities' (Ukrainian State Veterinary Service, Ukrainian Ministry of Healthcare, Ministry of Environment and Natural Resources Protection of Georgian National Food Agency) notification regarding the results of research will be organized due to the framework of the project; Practical recommendations for improvement of ecological

and epidemiological surveillance of bat diseases will be developed based on results obtained by all participants within the project and will be presented to Ukrainian and Georgian governments; The cooperation and joint scientific work between various scientific and diagnostic institutions in Ukraine, Georgia and USA.

Who are you?

National Scientific Center Institute of Experimental and Clinical Veterinary Medicine (NSC IECVM, Ukraine). Leading scientific institution of animal health in Ukraine, coordinates scientific research in dangerous pathogens in Ukraine. The NSC IECVM meets all Ukrainian State Sanitary BS&S rules (DSP 9.9.5.035-99) for working with especial dangerous pathogens (EDPs). NSC IECVM meets Ukrainian State Sanitary permission (No. 47-12, dated 6 April 2012) for working with EDPs. NSC IECVM has accreditation ISO 17025 (№241327, 14.08.2017). The NSC IECVM has successfully undertaken several international scientific projects concerning animal diseases, including highly pathogenic avian influenza and Newcastle disease viruses (e.g., STCU projects P-382 and P-382a Immunity concerning AI (2009-2013) USDA funds; P-444, P-444a, P-444b Wildlife epidemiology of HPAI and Newcastle disease (2010-2015) USDA funds, and P-568 Newcastle disease recombinant vaccines (2013-2016) DTRA funds; chlamydial infections in ruminants in Ukraine 2015-2017, Swiss National Science Foundation; two projects UP-4 and UP-10 in frame Ukraine Cooperative Biological Engagement Program (2016-2019). Laboratories have appropriate biosafety and biosecurity level (control access, video-surveillance, negative pressure air ventilation systems, PACS) and are equipped with PCR, DNA-RNA purification and preparation kits, electrophoresis (PAAG, AG), RRT PCR by BSC class 2, low-temperature chambers (refrigerators and freezers), equipment for ELISA, refrigerated centrifuge, water baths, autoclaves, drying chambers, equipment for serological studies, computers. The Commission for Bioethics and Animal Treatment has oversight of all projects involving animals or animal experimentation across the institute.

National Center for Disease Control and Public Health; Richard G. Lugar Center for Public Health Research (NCDC, Georgia) provides national leadership in preventing and controlling communicable and non-communicable diseases, disease surveillance, immunization, laboratory work, research, and responding to public health emergencies. The Lugar center is top-tiered institution in NCDC's laboratory network and serves as a reference laboratory of the Georgia's public health system. The Lugar Center's possesses a BSL-3 facility and BSL-2 space with following laboratories: Bacteriology, Virology, Molecular Biology/Genomics, Cell culture, Parasitology, Entomology, Vivarium, and the National Repository of human and animal EDPs. Since the Lugar Center is Georgia's only facility where the work with EDPs is conducted, safety and security are our primary concerns. We have robust engineering control in place, with double redundancy for all major facility systems. We have general and EDP-specific emergency response plans, planned emergency drills and identified first responders.

International collaborators: The National Center for Emerging and Zoonotic Infectious Diseases (NCEZID at the Centers for Disease Control and Prevention), The USGS National Wildlife Health Center (NWHC), Virginia Tech, US.

Expected results

Expected scientific results: Information about presence or absence of pathogens of potentially zoonotic infectious diseases to humans and animals in bats and assessment of pathogen diversity in bat populations of Ukraine and Georgia; phylogenetic characterization of the novel viruses and bacteria from insectivorous bats in Ukraine and Georgia. Expected non-scientific results: Training for personnel in safe wild animal capture, updates on new techniques

of specimen collection, and biosafety, implementation of high throughput laboratory techniques, GIS and its application to ecology and disease, and next generation sequencing analysis.

One Health Aspect: The results of laboratory research will be presented to Ukrainian State Veterinary Service (State Service for Food Safety and Consumer Protection), as well as to the Center of Diseases Control (Ukrainian Ministry of Healthcare) within the framework of One Health implementation. The results of laboratory research in Georgia will be presented to the Georgian Ministry of Agriculture and Georgian State Veterinary Service.

International, national, interdisciplinary, intersectoral cooperation. Experts from various fields (veterinary medicine, medicine, biology, molecular biology, zoology etc.) will be involved in the implementation of the project, both international and national levels. It will allow to build a sustainable network of experts in the study of bats, their pathogens and ecology.

Amount of work

- Improve practical skills of project participants and to develop/update/adapt SOPs for field and laboratory studies, trainings with will be provided by US-CDC, USGS (Year 1);
- Field expeditions for sampling of biological material from bats and collect data about study site, environment characteristics. (Year 1, Year 2, Year 3);
- Laboratory studies of collected biological material (PCR) (Year 1, Year 2, Year 3);
- Analyze, generalize and map all obtained field and laboratory results (Year 3);
- Check of scientific hypothesis (Year 2, Year 3);
- Presentation of preliminary and final results (oral or/and poster) on scientific conferences (Year 1, Year 2, Year 3); Publication of result into peer-reweave journals (Year 1, Year 2, Year 3).

Technical approach and methodology

Task for perform 1. Update and improve hands on skills of local staff by implementing harmonized standard operational procedures (SOPs) for field activities, collection and sampling of bats, laboratory procedures for the detection, typing and full genome characterization of the agents of interest. **(Year 1)**.

Subtask 1.1. Assessment of facilities and available infrastructure in Georgia and Ukraine where laboratory-based SOPs will be implemented. An inventory of available equipment, proper functioning and documentation of preventive maintenance will be checked. Information technology infrastructure availability of adequate computing capacity. Cold chain, storage space and adequate sample inventory. This activity should be conducted by a commission integrated by personnel of institutions responsible of implementation as well a representatives of the host institution (USCDC, USGS, IECVM, STCU and NCDC). IACUC approved protocols should be presented by participating institutions so field activities can start as soon as possible.

Subtask 1.2. A short seminar on harmonization of protocols for animal collection to submit to IACUC will take place after kickoff meeting, as well as on GIS techniques, trainings on how to obtain high quality data field and environmental variables. Brief tutorials on how to use some tools such as MERIS FR satellite-derived landscape features (Virginia Tech, CDC Luis Escobar Yoshinori Nakazawa).

Subtask 1.3. Specific trainings for IECVM and NCDC staff lead by US-CDC and USGS on best practices for wildlife field investigations, safe animal handling, specimen collection and transport, use of personal protective equipment and biosafety, cold chain management will be given

Subtask 1.4. Implement/update/harmonize SOPs for biosafety and biosecurity, laboratory testing, sequencing.

Subtask 1.5. Run first screening tests on samples recovered from shelters.

Subtask 1.6. Commence field expeditions and start sampling collection.

Task for perform 2. Continue field expeditions, SOPs trouble shootings, first analysis on positive rates and typing data. **(Year 1, Year 2, Year 3)**

Subtask 2.1. Continue collection of bats and biological specimens (NCDC, IECVM, US-CDC and USGS);

Subtask 2.2. Follow up on the data quality stored and data base management and curation (Virginia Tech and US-CDC).

Subtask 2.3. Follow up on correct execution of SOPs (QA/QC activities), review data of screening tests conducted on the first year, trouble shooting of laboratory procedures in case of necessary. Preliminary analysis of typing data (Activities coordinated by US-CDC, NCDC).

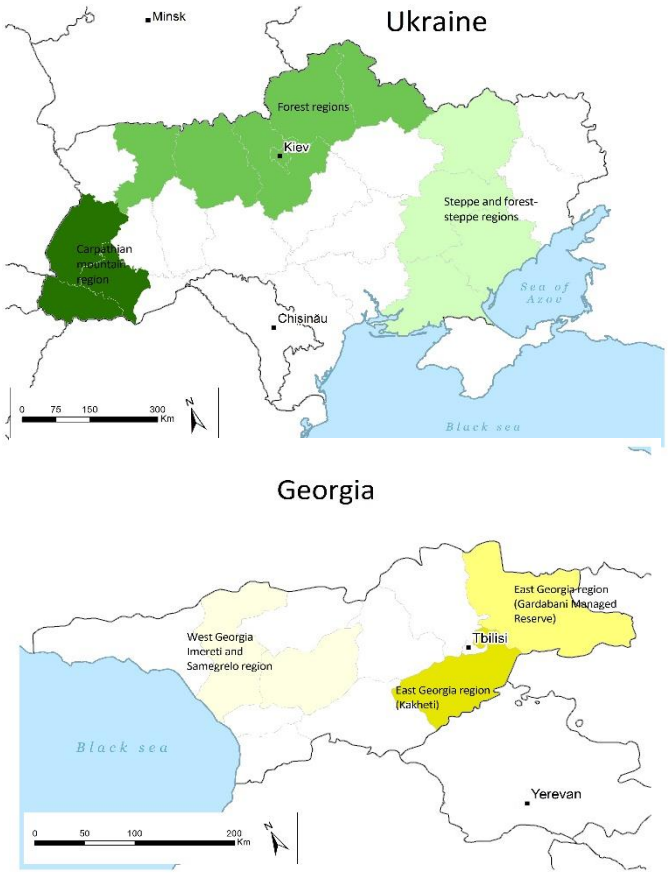

Fig. 4 Map of main sites in Ukraine and Georgia

Field activities will include bat capture, sampling, field work and biomaterial transportation to the laboratory in compliance to current biosafety/biosecurity and bio-ethic protocols. Bats will be trapped from different urban roosts and in wild ecosystems according to CDC/USGS best practices. The expected number of examined animals for three years of the project is about 850 in Ukraine and 650 in Georgia. Bats (in Ukraine and Georgia) will be mist-netted, captured and sampled in breeding season (May-July), during migration (August-September) and at the beginning of hibernation (November-December) in different geographic areas of Ukraine and Georgia. The field sites in Ukraine will located in three different regions: Carpathian mountain region (West Ukraine), forest regions (the North Ukraine), steppe and forest-steppe regions (the Eastern and Central Ukraine). There will three regions covered across Georgia: West Georgia Imereti and Samegrelo region, East Georgia region (Kakheti) and East Georgia region (Gardabani Managed Reserve) (Fig. 1).

Sampling. Two types of sampling (lethal and non-lethal) will be conducted during this investigation, no more than 120 bats in Georgia and no more than 150 bats in Ukraine during three years will be euthanatized. Nonlethal samplings will consist of collection of feces or rectal swabs, urine, oropharyngeal swabs will be obtained from live animals. Alternatively, collecting fresh feces from caves or roost floors may be used for overall screening of roosts. In the case of mono species colonies fresh feces will be used only. The species of bats in the colony will be clearly identified. In the case of several bat's species in the colony, samples will not be taken. Individually located faces will be sampled, which will be considered as the samples from one animal. When using fresh feces collected from the colony, the percentage of positive single feces samples will be evaluated. The last one will indicate about the total colony rate of infectivity. The size of the colony and the total number of animals will also be taken into account. Tissue will be collected as biological samples from dead or euthanized animals. Also we will collect GIS data about study site (position data, place of sampling, urban or wild place), environment characteristics

(landscape, weather conditions, season of year) and bat (number of animals, number of species in the place, physiological and health status, age, gender, etc.).

Sample transportation and storage. Common SOPs regarding transportation, storage, disinfection and utilization of samples according to CBEP and US CDC standards will be developed before the beginning of the project. Appropriate records for incoming biological specimens will be kept in PACS system in laboratories. Collected samples will be transported in dry shipping liquid nitrogen tanks. Frozen samples will be stored in -80^{0}C Ultra freezers in BSL 2 facility. All samples will be autoclaved after completion of all studies (at least 12 months after the project end date). Access (only for authorized persons) to all samples collected and data generated during the project will be up to and including at least 12 months after the project end date.

Landscape Characterization. To explore patterns of landscape characteristics in the study area, we will 16-day composite enhanced vegetation index (EVI) images at 250 m spatial resolution from the MODIS sensor (MOD13A1) on the Terra satellite (https://lpdaac.usgs. gov/data_access/data_pool).

Urbanization Estimation. We will use nighttime-light satellite-derived data as a proxy of levels of urbanization. Urbanization levels will be characterized in the form of a nighttime-light satellite image at ~0.75 km resolution matching our first fieldwork period. Data will be collected by the VIIRS sensor, at the Suomi NPP satellite (http://1.usa.gov/1FQvs5r).

Bat Diversity Estimation. Mammals will be captured across Ukraine and Georgia based on vegetation phenology and urbanization level, ranging from urban to dense forest, to achieve different species configurations and different levels of biodiversity.

Task for perform 3. Continue laboratory studies on biological material. **(Year 1, Year 2, Year 3)**.

Subtask 3.1. Processing of specimens (feces, urine and swab samples to detect nucleic acid of all agents of interest via PCR.

Subtask 3.2. Typing of positive samples through sequencing and phylogenetic inference.

Subtask 3.3. Full genome characterization through NGS data, standardization of an analytical pipeline (US-CDC trainings), phylogenetic analysis and application of comparative genomics approaches to determine relatedness to previously identified pathogens (Training activities lead by US-CDC).

Subtask 3.4. Implementation of data integration applications for special visualization of all generated data (sequence data, environmental variables), comparative genomic training. This activity will be lead by US CDC.

Subtask 3.5. Data integration continuation, development of ecological niche models (activity lead by Virginia Tech and US-CDC).

Laboratory studies. The project will focus on detection (PCR only) of Filoviruses, Paramyxoviruses, Lyssaviruses, Orthomyxoviruses, Brucella spp, Leptospira spp, Yersinia spp and in both countries and additional Coronaviruses (only in Ukraine). Study of coronaviruses in Georgia is included in other current project. During laboratory studies we will determine the presence of pathogens in the collected field samples. The presence of pathogens will be determined by genome detection in biological material. For detection all pathogens (except orthomyxoviruses) we will use SOP and methodology that will be provided CDC team. SOP and methodology for detection orthomyxoviruses will be provided USGS team.

Regarding viral agents, automated total RNA extraction for solid tissue pools (stool pellets, rectal swabs, lung, kidney, spleen, liver and intestines) per individual will be carried out. Total RNA pools made by mixing individual total RNA of 3-5 individuals of the same species and the same collection point will be prepared and subsequently screened by two to four independent real time RT-PCR or nested pan viral group end point PCR assays per each viral family or genus

of interest. Two assays would be used for coronaviruses, two for filoviruses, four for paramyxoviruses, two for lyssaviruses, and one for orthomyxoviruses.

All assay's designs use broadly reactive primers and probes to increase the detection sensitivity for new viral species. Samples in all positive pools will be re-screened by real time RT-PCR to determine the exact number of positive individuals per pool. Subsequent, individual end point RT-PCR in all positive individuals will target the amplification of highly informative genes or loci (2 to 3 kb), which then will be Sanger sequenced to determine the diversity spectrum for each viral family. The presence of pathogens will be calculated according to the results of laboratory studies. Statistical analyses will performed using general statistics methods (Pearson chi-square (χ^2) or Fisher's exact tests). Statistical analysis will be carried out using software program R (R Core Team 2019) or other.

Appropriate protocols for geocoding and importing data on the EDP's will be developed to ensure that data are captured in an appropriately formatted spreadsheet or comma-delimited text file. Once an array of disease data has been converted into digital GIS formats, will focus on concepts regarding spatial analysis, cartography, and methods for producing different types of maps for visualizing disease distributions. Topics covered will include: map projections, data selection, vector data analysis, raster analysis, data integration, data symbolization, and map design.

Place of project implementation and its technical equipment

In Ukraine the project will be implemented at the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" of the NAAS (83 Pushkinskaya Street, Kharkiv). Units of NSC "IECVM" that will be involved in the project are equipped with basic machinery for conventional PCR, qPCR, low-temperature refrigerators, class 2 biosafety rooms, centrifuges with cooling, water baths, autoclaves, drying chambers and computer for data analysis. In addition, all rooms involved in the project have ventilation with HEPA filters providing a pressure difference in accordance with the BSL2 + classification.

In Georgia the project will be implemented at **National Center for Disease Control and Public Health; Richard G. Lugar Center for Public Health Research (NCDC, Georgia)** provides national leadership in preventing and controlling communicable and non-communicable diseases, disease surveillance, immunization, laboratory work, research, and responding to public health emergencies. The Lugar center is top-tiered institution in NCDC's laboratory network and serves as a reference laboratory of the Georgia's public health system. The Lugar Center's possesses a BSL-3 facility and BSL-2 space with following laboratories: Bacteriology, Virology, Molecular Biology/Genomics, Cell culture, Parasitology, Entomology, Vivarium, and the National Repository of human and animal EDPs. Since the Lugar Center is Georgia's only facility where the work with EDPs is conducted, safety and security are our primary concerns. We have robust engineering control in place, with double redundancy for all major facility systems. We have general and EDP-specific emergency response plans, planned emergency drills and identified first responders.