BIOLOGICAL ENGAGEMENT PROGRAMS: REDUCING THREATS AND STRENGTHENING GLOBAL HEALTH SECURITY THROUGH SCIENTIFIC COLLABORATION

EDITED BY: Jeanne M. Fair, Hillary H. Carter and Nathan Wolfe
PUBLISHED IN: Frontiers in Public Health and Frontiers in Microbiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-273-6
DOI 10.3389/978-2-88945-273-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

BIOLOGICAL ENGAGEMENT PROGRAMS: REDUCING THREATS AND STRENGTHENING GLOBAL HEALTH SECURITY THROUGH SCIENTIFIC COLLABORATION

Topic Editors:
Jeanne M. Fair, Los Alamos National Laboratory (DOE), United States
Hillary H. Carter, U.S. Department of State, United States
Nathan Wolfe, Metabiota, United States

Lone Saiga Antelope in Kazakhstan.
The photo is from a collaborative research project between Kazakhstan and the U.S. investigating recent outbreaks of an unidentified infectious disease in the saiga. Image credit: Dr. Jason Blackburn at the University of Florida.

Biological engagement programs are a set of projects or activities between partner countries that strengthen global health security to achieve mutually beneficial outcomes. Engagement programs are an effective way to work collaboratively towards a common threat reduction goal, usually with a strong focus on strengthening health systems and making the world a safer place. Cooperative programs are built upon trust and sharing of information and resources to increase the capacity and capabilities of partner countries. Biological engagement programs reduce the threat of infectious disease with a focus on pathogens of security concern, such as those pathogens identified by the U.S. Government as Biological Select Agent and Toxins. These programs seek to develop technical or scientific relationships between countries to combat infectious diseases both in humans and animals. Through laboratory biorisk management, diagnostics,

pathogen detection, biosurveillance and countermeasure development for infectious diseases, deep relationships are fostered between countries. Biological engagement programs are designed to address dual-use issues in pathogen research by promoting responsible science methodologies and cultures. Scientific collaboration is a core mechanism for engagement programs are designed to strengthen global health security, including prevention of avoidable epidemics; detection of threats as early as possible; and rapid and effective outbreak response.

This Research Topic discusses Biological Engagement Programs, highlighting the successes and challenges of these cooperative programs. Articles in this topic outlined established engagement programs as well as described what has been learned from historical cooperative engagement programs not focused on infectious diseases. Articles in this topic highlighted selected research, trainings, and programs in Biological Engagement Programs from around the world. This Topic eBook first delves into Policies and Lessons Learned; then describes Initiatives in Biosafety & Biosecurity; the core of this work documents Cooperative Research Results from the field; then lastly the Topic lays out potential Future Directions to the continued success of the World's cooperative science in reducing the threat of infectious diseases.

Citation: Fair, J. M., Carter, H. H., Wolfe, N., eds. (2017). Biological Engagement Programs: Reducing Threats and Strengthening Global Health Security Through Scientific Collaboration. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-273-6

Table of Contents

Future Directions

frontiers in Public Health

EDITORIAL
published: 12 July 2017
doi: 10.3389/fpubh.2017.00148

Editorial: Biological Engagement Programs: Reducing Threats and Strengthening Global Health Security Through Scientific Collaboration

*Jeanne M. Fair**

Biosecurity and Public Health, Los Alamos National Laboratory (DOE), Los Alamos, NM, United States

Keywords: cooperative engagement, emerging diseases, biothreat, Global Health Security Agenda, One Health

The Editorial on the Research Topic

Biological Engagement Programs: Reducing Threats and Strengthening Global Health Security Through Scientific Collaboration

It is often said about infectious diseases that a "threat anywhere is a threat everywhere," and the recent outbreaks of Ebola in West Africa and Zika virus in South America have proven that pathogens know no borders. Not only are they transboundary, pathogens do not discriminate who they infect. In addition to the natural increase in emerging zoonotic infectious diseases worldwide due to changing environmental conditions and globalization, the use of infectious diseases as warfare agents is a threat in today's world. Early detection remains one of the best ways to prevent small outbreaks becoming epidemics and pandemics. Accurate diagnosis, detection, and reporting of diseases are important components of mitigating outbreaks, and biosurveillance remains the top tool in our toolbox. While vaccines have been important for controlling more common infectious virus diseases, they are less feasible for less common diseases, emerging pathogens, and rapidly evolving microbes. Due to globalization and increased travel, emigration, and migration, biosurveillance is critical throughout the world, not just in pockets of more developed regions.

Building up the capabilities and capacities for biosurveillance is a global challenge. Cooperative biological engagements help address biosurveillance and biosafety gaps in capabilities and reduce threats worldwide, by strengthening biosurveillance globally in a number of ways. The first is in assisting countries and regions to increase their technical expertise for detecting, diagnosing, and reporting on rapidly changing and emerging infectious diseases. Second, cooperation can help strengthen the biosafety and biosecurity of laboratories around the world. Third, biosurveillance can be strengthened by understanding the best strategies for biosurveillance planning, and the potential epidemiology of a disease system within a region. In these instances, collaborative research comes into play to help scientists understand a disease system in the environment and devise the most effective strategy for detecting outbreaks. The articles in the Frontier Topic "Biological Engagement Programs: Reducing Threats and Strengthening Global Health Security Through Scientific Collaboration" cover each of these primary areas of international collaboration. This topic brings together 148 authors from over 25 countries with the shared mission of reducing the threat of infectious diseases.

Reducing the threat of a nefarious use of pathogens on any human or animal population is a top priority for global security. Specifically, the Global Health Security Agenda (GHSA) is "an effort by nations, international organizations, and civil society to accelerate progress toward a world safe and secure from infectious disease threats; to promote global health security as an international priority; and to spur progress towards reducing infectious diseases" (1). Working with partner countries around the world, the GHSA will be focused on mitigating the impact of naturally occurring outbreaks and intentional or accidental releases of dangerous pathogens; assisting countries to rapidly detect and transparently report outbreaks when they occur; and employing

OPEN ACCESS

Edited by:
Rustam Aminov, University of Aberdeen, United Kingdom

Reviewed by:
Svetlana Khaiboullina, Whittemore Peterson Institute, United States

***Correspondence:**
Jeanne M. Fair
jmfair@lanl.gov

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *22 March 2017*
Accepted: *12 June 2017*
Published: *12 July 2017*

Citation:
Fair JM (2017) Editorial: Biological Engagement Programs: Reducing Threats and Strengthening Global Health Security Through Scientific Collaboration. Front. Public Health 5:148. doi: 10.3389/fpubh.2017.00148

an interconnected global network that can respond rapidly and effectively. The most important component of the GHSA is international cooperation (Galloway et al.). Galloway et al. present a review of the GHSA and the importance of being proactive in the new era of globalization. In addition, Standley et al. give a history of Cooperative Threat Reduction programs and how cooperative bioengagements can assist in the implementation of the International Health Regulations.

Cooperative engagements and collaborations across borders help foster open communication and sharing of data. Being aware of the transboundary nature of pathogens helps break down the barriers to sharing information between countries, and cooperative engagement programs are designed to build a foundation of trust that can help lessen potential negative aspects of sharing data such as economic or political consequences. The fight against infectious diseases is shared by humanity; reducing individual infections and outbreaks of zoonotic diseases in humans, agricultural animals, and wildlife is a shared goal across the world. Fair et al. present a model for measuring the return on relationships of collaborations and the resulting networks of people that remain in place after trainings or projects are complete.

BIOSAFETY AND BIOSECURITY CHALLENGES

Sampling and laboratory analysis for infectious diseases requires a certain amount of infrastructure and unique skills in molecular techniques in virology and bacteriology. Samples may have to be cultured and saved for future reference, and the microbiology environment for working with such pathogens must be both safe and secure. Best practices for biosafety and biosecurity are often learned through previous mistakes in the field and laboratory. Sharing these lessons learned is a critical factor in strengthening the biosafety and biosecurity environment in laboratories around the world. Khan et al. discuss biosafety initiatives and gaps in the BMENA region. In addition, Al Jewari and Koblentz share how to strengthen biosecurity in Iraq and the development of an Iraqi National Biorisk Management System.

"ONE WORLD, ONE HEALTH" UNIFICATION

The One World, One Health (OWOH) agenda is based on the foundation that most pathogens continually circulate in animal species and that there is a constant interplay between agricultural animals, wildlife, the environment, and humans. Therefore, the OWOH agenda is focused on surveillance, biosecurity, and biodiversity developed too limit infectious agents in a synergistic manner with animals, humans, and the environment. The unified and holistic approach to OWOH health was established in 2004 at a New York meeting where 12 principles were defined for multidisciplinary and integrated approaches to health. Over the last decade, the One Health approach has been applied to disease situations around the world and while some sociological challenges have been identified (2, 3), many success stories can also be told. As a common outcome of biological cooperative engagement projects, Ministries of Health in over 25 countries worldwide have worked closely with Ministries of Agricultural, leading to more communication, sharing, and cooperation on zoonotic diseases across disciplines. Several papers in this Frontiers Topic review efforts to increase capabilities for biosurveillance such as developing genomic capabilities for detecting pathogens by Cui et al.

HOW CAN RESEARCH HELP ADDRESS THE CHALLENGE OF OUTBREAKS?

Many of the papers in this Frontiers Topic highlight collaborative research on infectious pathogens of security concern. For example, Bartholomew et al. review the history of building infectious disease research programs with countries of the Former Soviet Union. Scientific research on infectious diseases often focuses on reductionism, or understanding the molecular and physiological mechanisms of host–pathogen interactions. Research may also focus on a higher scale of understanding the disease "system." Several papers in this collection highlight studies for understanding the diseases systems, such as Kokashvili et al. reporting on Vibrio species in the aquatic environment of Georgia, and the epizootology of Lumpy Skin disease in livestock in Azerbaijan by Zeynalova et al.

Cooperative biological engagement research tends to focus on the higher system-level scale since its objective is to increase the effectiveness of biosurveillance. For example, understanding a disease "system" such as Middle East Respiratory Syndrome coronavirus in the Middle East can lead to insights into the transmission events as well as better detection and possible mitigations to stop the infectious cycle. Understanding a disease system may sometimes require gathering information that may appear irrelevant to the disease, but may be critical for comprehending its spread. For example, mapping the distribution of bat species in a region and their migratory patterns can provide vital clues as to how and why disease outbreaks keep occurring or are emerging. Host range and host heterogeneity are important aspects of a disease system, as is identifying dead end hosts, regular host, and potential "super-spreaders."

With limited monetary resources for biosurveillance, efforts need to be as directed and thoughtful as possible in order to be cost effective and successful. Developing the best strategy for biosurveillance requires knowledge of disease systems and that requires methodical and hypothesis-based scientific research. The last and most critical step is then applying the knowledge learned from scientific studies to inform policies. Blackburn et al. share examples of the applications of research on infectious diseases to policies for mitigating and responding to disease outbreaks. Two other papers by Horn and Hay et al. discuss the challenges of doing cooperative research in austere environments with take-home lessons for all future cooperative science engagements [Hay et al.; Horn].

GLOBAL CHALLENGES REQUIRE GLOBAL COLLABORATION

Rates of evolution of phenotypic traits in species vary widely in a continuum of slow to rapid evolution. Species may adapt to

environmental changes differently and in the instance of climate change, species that are not able to adapt to a rapidly changing environment may be worse off than species that can. Clear evidence is mounting that changes in mean temperature or climate variability are increasing infectious disease risk globally (4, 5). Cooperation will continue to be important as vectors, hosts, and pathogens shift their ranges and seasonality.

Selection pressures may also force rapid evolution in species with short generation times, such as microbes. Antimicrobial resistance (AMR) is an example of rapid evolution in response to selection pressures, primarily in response to antimicrobial drugs. AMR is now considered a major global threat to public health (6, 7). In 2015, the World Health Assembly endorsed a global action plan to tackle AMR, with a primary focus on antibiotic resistance (8). AMR is occurring everywhere in the world, compromising the ability to treat infectious diseases with life-saving drugs of the past such as penicillin. The goal of the global action plan is to ensure, for as long as possible, continuity of successful treatment and prevention of infectious diseases with effective and safe medicines that are quality-assured, used in a responsible way, and accessible to all who need them. Again, because antimicrobial selection pressures may vary between country and region, international collaboration is required to tackle the challenge of increasing antimicrobial-resistant pathogens. Antimicrobial-resistant microbes also know no borders.

The scientists and authors who have come together in this Frontiers Topic on cooperative biological engagements have a shared passion and mission for both reducing the threat of infectious diseases, and international collaboration and coordination. Coming together across the globe allows for a greater diversity of ideas that then leads to more innovation and creative problem solving. Shared insights from direct experiences and research increase the ability to reduce infectious disease outbreaks. Reducing outbreaks, epidemics, and pandemics potentially saves thousands of lives. While it has always been difficult to "prove a negative" for the effectiveness of programs such as cooperative biological engagements, the success stories are there and the scientific research that comes from such programs is invaluable. We are indebted to the work of everyone involved in such programs around the world, and especially to the authors contributing to this special Frontiers Topic.

AUTHOR CONTRIBUTIONS

This is a single author paper by the primary Editor for the Topic "Biological Engagement Programs: Reducing Threats and Strengthening Global Health Security Through Scientific Collaboration." This editorial that introduces the Topic was completely written by JF.

REFERENCES

1. CDC. *Global Health Security Agenda*. (2017). Available from: https://www.cdc.gov/globalhealth/security/
2. Woldehanna S, Zimicki S. An expanded One Health model: integrating social science and One Health to inform study of the human-animal interface. *Soc Sci Med* (2015) 129:87–95. doi:10.1016/j.socscimed.2014.10.059
3. Hinchliffe S. More than one world, more than one health: re-configuring interspecies health. *Soc Sci Med* (2015) 129:28–35. doi:10.1016/j.socscimed.2014.07.007
4. Altizer S, Ostfeld RS, Johnson PTJ, Kutz S, Harvell CD. Climate change and infectious diseases: from evidence to a predictive framework. *Science* (2013) 341:514–9. doi:10.1126/science.1239401
5. Rodó X, Pascual M, Doblas-Reyes FJ, Gershunov A, Stone DA, Giorgi F, et al. Climate change and infectious diseases: can we meet the needs for better prediction? *Clim Change* (2013) 118:625–40. doi:10.1007/s10584-013-0744-1
6. Ferri M, Ranucci E, Romagnoli P, Giaccone V. Antimicrobial resistance: a global emerging threat to public health systems. *Crit Rev Food Sci Nutr* (2015) 57(13):2857–76. doi:10.1080/10408398.2015.1077192
7. Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, et al. The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect* (2015) 6:22–9. doi:10.1016/j.nmni.2015.02.007
8. WHO. *Global Antimicrobial Resistence Action Plan*. (2017). Available from: http://www.who.int/antimicrobial-resistance/global-action-plan/en/

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 08 January 2016
doi: 10.3389/fpubh.2015.00276

Applying Science: Opportunities to Inform Disease Management Policy with Cooperative Research within a One Health Framework

Jason K. Blackburn[1,2]*, *Ian T. Kracalik*[1,2] *and Jeanne Marie Fair*[3]

[1] *Spatial Epidemiology and Ecology Research Laboratory, Department of Geography, University of Florida, Gainesville, FL, USA,* [2] *Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA,* [3] *Cooperative Biological Engagement Program, Defense Threat Reduction Agency, Fort Belvoir, VA, USA*

The ongoing Ebola outbreak in West Africa and the current saiga antelope die off in Kazakhstan each represent very real and difficult to manage public or veterinary health crises. They also illustrate the importance of stable and funded surveillance and sound policy for intervention or disease control. While these two events highlight extreme cases of infectious disease (Ebola) or (possible) environmental exposure (saiga), diseases such as anthrax, brucellosis, tularemia, and plague are all zoonoses that pose risks and present surveillance challenges at the wildlife-livestock–human interfaces. These four diseases are also considered important actors in the threat of biological terror activities and have a long history as legacy biowarfare pathogens. This paper reviews recent studies done cooperatively between American and institutions within nations of the Former Soviet Union (FSU) focused on spatiotemporal, epidemiological, and ecological patterns of these four zoonoses. We examine recent studies and discuss the possible ways in which techniques, including ecological niche modeling, disease risk modeling, and spatiotemporal cluster analysis, can inform disease surveillance, control efforts, and impact policy. Our focus is to posit ways to apply science to disease management policy and actual management or mitigation practices. Across these examples, we illustrate the value of cooperative studies that bring together modern geospatial and epidemiological analyses to improve our understanding of the distribution of pathogens and diseases in livestock, wildlife, and humans. For example, ecological niche modeling can provide national level maps of pathogen distributions for surveillance planning, while space-time models can identify the timing and location of significant outbreak events for defining active control strategies. We advocate for the need to bring the results and the researchers from cooperative studies into the meeting rooms where policy is negotiated and use these results to inform future disease surveillance and control or eradication campaigns.

Keywords: disease surveillance, disease modeling, anthrax, brucellosis, plague, tularemia, one health

OPEN ACCESS

Edited by:
Joav Merrick,
Ministry of Social Affairs, Israel

Reviewed by:
Paras Jain,
Albert Einstein College of Medicine, USA
Seter Siziya,
Copperbelt University, Zambia

***Correspondence:**
Jason K. Blackburn
jkblackburn@ufl.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *19 June 2015*
Accepted: *07 December 2015*
Published: *08 January 2016*

Citation:
Blackburn JK, Kracalik IT and Fair JM (2016) Applying Science: Opportunities to Inform Disease Management Policy with Cooperative Research within a One Health Framework. Front. Public Health 3:276. doi: 10.3389/fpubh.2015.00276

INTRODUCTION

The recent Ebola outbreak in West Africa (1, 2) has been a shocking reminder of the ever present risk of rapidly spreading disease outbreaks and the reality of the difficulties involved in outbreak response (3) and surveillance. That outbreak, resulting in more than 10,000 human deaths (and still ongoing at the time of this writing), highlights the severity that re-emerging diseases can pose.

The difficulties in identifying the potential zoonotic source of this infection (4) highlight the importance of understanding interactions at the human–wildlife interface (5) and of the importance of wildlife surveillance. The ongoing saiga antelope, *Saiga tatarica* die off in Kazakhstan, where more than 100,000 antelope have died in <8 weeks[1] (representing approximately 1/2 of all remaining saiga), presents another example of a severe loss of animals to unknown environmental contamination or pathogen exposure. While the ongoing Ebola outbreak and saiga die off represents the extreme of outbreak consequences (high human or wildlife mortality), several other important zoonoses have been re-emerging or maintaining with high incidence in known endemic areas. Diseases such as anthrax, brucellosis, tularemia, and plague are all zoonoses that pose risks and present surveillance challenges at the wildlife-livestock–human interfaces. These four diseases are also considered the most important pathogens for use in biological terror activities and have a long history as legacy biowarfare pathogens. Inter-specific transmission of each of these diseases demands that surveillance should include coordination between veterinary and human health personnel. This paper will review recent studies done cooperatively between American and institutions within nations of the Former Soviet Union (FSU) focused on spatiotemporal, epidemiological, and ecological patterns of these four zoonoses. These cooperative studies were all funded by the Defense Threat Reduction Agency's Cooperative Biological Engagement Program (or previous iterations of the program such as the Biological Threat Reduction Program). The goal of those efforts was to bring local and American scientists together to apply contemporary geospatial and epidemiological techniques to the issues of zoonotic disease persistence and transmission in the FSU with an emphasis on pathogens on the US Federal Select Agent Program pathogen list[2].

These projects were designed to be aimed at understanding disease spatiotemporal patterns and ecology. While the disease systems, study sites, and geospatial techniques differed, each of the studies highlighted here are related in that they applied geospatial modeling techniques to better understand disease patterns in humans, livestock, or wildlife. From these examples, we identify how study results may be use to inform national and international policy making to improve disease surveillance and inform control strategies. Broadly, these techniques rely on surveillance-derived datasets describing either case reports or serological evidence of disease presence at a spatial resolution smaller than the national level; most data were either farm or village locations mapped as GPS coordinate pairs or data aggregated to the rayon (district equivalent). Across the studies that we survey in this paper, it is noteworthy to point out a significant DTRA investment in data development for these projects in terms of data access, data compilation, and personnel time required. Such efforts are instrumental in cooperative biological engagements and are an important and often undervalued part of disease ecology studies.

This paper illustrates how these techniques can inform disease surveillance, control efforts and impact policy. Our focus is to posit ways to apply science to disease management policy and actual management or mitigation practices. As part of this, we emphasize the importance of data sharing between human and veterinary health professionals and suggest actionable ways of improving data sharing. The objective of this paper were to review four diseases (anthrax, brucellosis, tularemia, and plague), that are important to cooperative biological engagement programs, in relation to published research studies and the resulting impact on policy and management decisions.

[1]http://www.mnn.com/earth-matters/animals/stories/why-are-saiga-antelope-dying-in-record-numbers

[2]http://www.selectagents.gov/

PATHOGENS AND POLICIES

Anthrax

Globally, anthrax is an important zoonosis with rapid onset and high mortality in wildlife and livestock, a cause of secondary human cases, and a security risk as a bioterror agent (6). The disease, caused by the Gram-positive, spore-forming bacterium *Bacillus anthracis,* can exert significant impacts on wildlife and livestock populations (7, 8). Human cases are most commonly associated with slaughtering infected animals (9). However, anthrax ecology and transmission remain poorly understood and understudied (10). Broadly, outbreaks in enzootic regions arise in specific environmental conditions (11), such as semi-arid grasslands and steppes, and at times of seasonal transitions in climate (12–14). This is true for livestock and wildlife populations. Reports of wildlife cases are becoming more common in North America. Wildlife outbreaks are regular in white-tailed deer, *Odocoileus virginianus*, in Texas (10) and bison, *Bison bison* ssp., in Canada (15). Globally, livestock outbreaks have also been associated with contaminated animal feed (16).

Anthrax occurs nearly worldwide with the heaviest livestock and human disease burden in resource limited countries (17). Animal cases occur across much of the globe, but human cases are most concentrated in Central Asia, Southern Africa, and the Caucasus. Over the last two decades, based on reviews from Promed Mail, Kyrgyzstan had some of the highest reported human case numbers. Additionally, Turkey (18) and Georgia (19) have both reported high numbers of human cases; Georgia has seen significant increases in human anthrax in the last few years (20). Disease control in these areas requires approaches that provide livestock control, such as vaccination campaigns and increased animal surveillance (21) and education programs targeting animal handlers and local slaughter operations.

Anthrax in Azerbaijan and Georgia

A recent cooperative study by Kracalik et al. (22) examined the spatial patterns of human anthrax in Azerbaijan looking at three time periods: Soviet (1984–1991), post-Soviet (1992–1995), and post-independence following livestock vaccination campaigns (1996–2010). Generally, the rate of human anthrax increased from the Soviet to the post-Soviet periods, which was most likely due to the drastic decrease in funding and changes in livestock management associated with Azerbaijani independence. From the post-Soviet through the post-independence period, there was a drastic decrease in the overall human incidence rate and a geographic shift in the concentration of reporting. These results suggest that livestock-associated human anthrax can be controlled

with livestock vaccination campaigns. Furthermore, these findings indicate that surveillance cannot focus solely on areas of historic disease presence, but rather needs to be dynamic and sensitive to changes in livestock distributions and socioeconomic shifts associated with agricultural production.

Like Azerbaijan, Georgia has seen an increase in human anthrax cases over the last decade, characterized by a drastic increase over the past 5 years (20). In recent years, Georgia has seen some of the highest rates of human anthrax globally. Cooperative research has shown the during the period 2000–2009, clusters of human anthrax cases in eastern and west central Georgia were associated ecological conditions that promote pathogen persistence (19). Consistent with research in Azerbaijan, these studies identified spatial heterogeneity across the Georgia landscape suggesting control efforts should be targeted to prioritize high risk areas. One major contrast between the two countries is the change in livestock vaccination policy, while Azerbaijan maintained vaccination through the last decade, Georgia ended compulsory vaccination in 2007, which lead to a drastic decrease in the number of animals vaccinated. This compulsory policy was nominally reinstated in 2012 following a historical rise in cases (20), though the total coverage of animals remains low. In both countries, anthrax surveillance remains highly anthropocentric, making it difficult to identify livestock populations at greatest risk. The use of spatial analyses in these studies provides a starting point for identifying areas where livestock surveillance should be prioritized.

Another important finding of the cooperative research in Georgia is related to the human populations at risk. Classically, anthrax is considered a rural disease. However, the recent increase in Georgian anthrax has been associated with an increase in peri-urban and urban dwellers likely associated with the selling of contaminated meat (19, 20). From a policy perspective, these results indicate a need for increased inspection and regulation of meat markets and sources of meat. Likewise, marketplaces may serve as points of outreach for public health education campaigns directed at both meat consumers and meat producers.

Anthrax in Kazakhstan

The Central Asian Steppe has a long history of livestock and human anthrax (23). Using data compiled from 1930 to 2006, several cooperative studies have increased our knowledge of the spatiotemporal, and ecological distribution of anthrax in Kazakhstan (21, 24–27). These studies have implemented predictive modeling approaches to map the distribution of anthrax and *B. anthracis* in Kazakhstan. Research by Joyner et al. (26) used livestock anthrax outbreak locations reported between 1960 and 2000 to develop an ecological niche model. A subsequent study refined those predictions to identify areas of anthrax risk by using a combination of spatial analysis and generalized linear modeling (21). Both the ecological niche and risk models identify areas that can be used to define surveillance areas, with risk models best used to prioritize areas for preemptive annual livestock vaccination campaigns. From a policy perspective, passive surveillance zones require laboratory infrastructure and veterinary training to identify and test for anthrax should spring or summer time livestock die offs present. These studies illustrate specific examples of how maps of pathogens (ecological niche models) or disease risk (predicting clusters) can be used to prioritize surveillance and control. Such approaches can be introduced into other countries where anthrax surveillance and control remains a challenge.

Anthrax in Ukraine

Like Kazakhstan, Ukraine has a long documented history of livestock and human anthrax (28). Two cooperative studies have recently been published that illustrate the changing distribution of livestock anthrax in Ukraine. First, Bezymennyi et al. (28) examined spatial patterns of livestock anthrax (1913–2012) in pre- and post-Soviet Ukraine. Like many countries, during the last 50 years, Ukraine saw a drastic reduction in the overall reports of anthrax and a contraction of the geographic area where anthrax persists. Following the dissolution of the Soviet Union, livestock anthrax increased briefly (1992–1997). During the last 17-years, there has been a reduction in reporting and a contraction of anthrax foci on the landscape, although compulsory vaccination in Ukraine extends only to state owned livestock operations. Independent livestock owners are less likely to vaccinate, as they bear the burden of cost. A recent outbreak in eastern Ukraine illustrated the ongoing risk when an unvaccinated cow died of anthrax, and was subsequently fed to a domestic dog that also succumbed to infection (29). Furthermore, there was an attempt to sell the infected meat at a market, which could have resulted in human cases had the public health service not halted its sale (28). From a policy perspective, these studies identify the need to examine definitions of compulsory vaccination and the cost benefits of requiring private owners to bear vaccination costs. Similar to the situation in Georgia, these studies also identify the need to evaluate meat inspection processes and to meet regulatory infrastructure to better identify potential sources of contamination meat being sold to the public.

An additional published collaborative study highlights the importance of including wildlife and surveillance for zoonotic diseases (30). In that study, the serological survey of wild boar collected from hunter check stations confirmed that at least some boars are exposed to *B. anthracis* in Ukraine. Most interesting was the fact that positive boars were identified in proximity to historical hotspots of anthrax but not directly overlapping with them. From a policy perspective, these results suggest surveillance should not be limited to livestock and should include wildlife populations. A similar strategy could be employed in Kazakhstan where there is potential overlap between saiga antelope and livestock (31) and may also be useful in the Caucasus, particularly Georgia, where livestock anthrax has been increasing.

Brucellosis

Brucellosis is one of the most widespread zoonotic diseases worldwide and is regarded as an emerging and re-emerging threat to public and veterinary health worldwide (32). Controlling brucellosis in humans is dependent upon limiting or reducing infection in livestock. Despite the availability of effective livestock vaccines for *Brucella spp.*, the disease continues to pose a global public health threat. Regions most heavily burdened by the disease include countries of the Mediterranean, Central Asia, Middle East, Latin America, Sub-Saharan African, and Balkan Peninsula (33).

The causative agents of the disease are a group of pathogenic bacteria in the genus *Brucella*, which primarily infect animal reservoirs. Humans are often secondarily infected through the consumption of unpasteurized dairy products or coming into contact with infected animals during animal husbandry or meat processing (32). The primary agents of infection in humans are *Brucella abortus* (cattle), *Brucella melitensis* (sheep and goats), *Brucella suis* (swine), and *Brucella canis* (dogs) (34).

Brucellosis in Azerbaijan

The disease was first reported from Azerbaijan in 1922 and quickly spread to more than two-thirds of the rayons in <30 years (35). Recent governmental changes brought on by the collapse of the Soviet Union have likely contributed the persistence of the disease, due primarily to decreased funding for surveillance and eradication programs (33). A recent cooperative study evaluated changes in the distribution of human brucellosis over each of three 5-year intervals from 1995 to 2009 (36). That study documented rayon-level disease persistence in humans that can direct contemporary surveillance efforts. As was suggested for Georgia, these data can be used to evaluate the cost-effectiveness and human health impacts of livestock disease control programs.

Brucellosis in Georgia

Brucellosis is an endemic livestock disease in Georgia with a relatively high human burden (37). In a recent study, data from a contemporary livestock serological survey were used to estimate true disease prevalence in a Bayesian framework comparing each of three important areas within Georgia (Imereti – west central, Kvemo Kartli – southern, and Kakheti – eastern) (38). In total, these three regions represent approximately 45% of George's milk production and total livestock population. Results from this study of livestock match a recent study of human brucellosis rates in Georgia (39). From a policy perspective, the results of the livestock surveillance study can be used to inform brucellosis control and eradication campaigns focusing first on the areas with greatest livestock brucellosis prevalence. It is likely that control in these areas would result in immediate and measurable improvements in human brucellosis incidents. As was illustrated in the anthrax work of Kracalik et al. (20, 22) in Azerbaijan, surveillance data from humans and animals could be analyzed in a one health framework that directly measure the impact of livestock control on human disease burden.

Plague

Plague is a flea borne zoonosis caused by the Gram-negative bacterium *Yersinia pestis* (40). Since the onset of the most recent pandemic, which started in China during the mid-nineteenth century, the geographic range of plague has greatly expanded (40). Classically, *Y. pestis* is maintained a sylvatic transmission cycle between partially resistant rodent hosts and adult hematophagous fleas (40) and foci can be maintained indefinitely in enzootic or maintenance cycles as long as sufficient numbers of rodent hosts and flea vectors are present. Natural plague reservoirs are active in Asia, and parts of the Russian Federation (41). Human plague cases have recently reemerged in this region (42).

Plague in Azerbaijan

Morris et al. (43) used historical maps of plague hosts derived annually between 1972 and 1985 to identify areas on the Azerbaijani landscape were plague carrying mammal densities were high and stable across years. The study digitized historical maps into active GIS layers and applied modern spatial analyses to evaluate areas of disease stability. The goal of the effort was to identify environmental conditions and geographic areas of historical sampling that may identify priority areas for contemporary sampling. In the years following Soviet independence, funds for plague surveillance were limited creating a gap in surveillance needing to be filled. That study identified a few key areas on the Azerbaijani landscape where plague may reemerge today. Beyond identifying those locations, Morris et al. (43) also identified which of those areas were in closest proximity or directly overlapping with increasing human populations. A similar effort field survey in neighboring Iran went a step further and use serological screening of dogs and small rodents and confirmed that historically defined foci could be active as many as two decades after the most recent zoological surveillance (44). From a policy perspective, the Morris et al. (43) all study in Azerbaijan can be used to prioritize exploratory surveillance in historically defined plague foci using serological testing or PCR-based methods. The areas identified as having increasing human population can further be used to prioritize those areas most important for surveillance. In this example, those areas with historically high rodent populations that saw little development until recently should be considered areas of highest likelihood of overlap between those contemporary rodent populations and human encroachment into those habitats.

Tularemia

Francisella tularensis, the causative agent of tularemia, is a zoonotic, Gram-negative bacterium that is broadly distributed across the Northern Hemisphere (45). Human exposure may occur through various pathways including arthropod bites, ingesting contaminated food products or liquids, inhaling aerosolized bacteria, or handling infected animals (45). Despite a global decline in reported human cases (46), tularemia has recently (re)emerged in several countries [summarized by Hightower et al. (47)] Historically, outbreaks in the FSU were linked to small mammals and arthropods (ticks), possibly related to increases host or vector population abundance or density or water-borne outbreaks. Tularemia foci were previously described in the 1960s across a limited geography in the south of Ukraine where several arthropods and small mammals were recognized as competent vectors and hosts (48; 49). However, contemporary characterizations of the spatial distribution and composition of vectors and hosts remains incomplete and should remain a priority in countries with known tularemia outbreaks (47).

Tularemia in Ukraine

Tularemia has a long history in Ukraine. In an effort to understand the historical distribution and identify possible areas of contemporary surveillance, Hightower et al. (47) mapped the spatial patterns of historical *F. tularensis* isolates from the Ukrainian Central Sanitation and Epidemiological Station (CSES; now Ukrainian Center for Disease Control) and tested for space-time clusters

on a database spanning more than 60 years. That study identified several historical foci that may serve as areas of persistence where disease reemergence is likely in humans. Additionally, that study defined tick vector and mammalian host species that should be priorities for sylvatic surveillance efforts. Hightower (50) use those data to construct small mammal and tick species-specific ecological niche models to estimate the potential geographic distribution of the pathogen across Ukraine. From a policy perspective, these studies provide Ukraine with specific local areas where disease surveillance should be focused. The space-time clusters defined can also serve as a baseline for comparing contemporary surveillance results. Additionally, Hightower et al. (47) identified areas where potential environmental exposure to contaminated crops may serve as an important transmission source that may not be detected from small mammal surveillance. These areas require additional infrastructure for testing such samples in the absence of human cases.

A Call for One Health Strategies for Improved Disease Surveillance and Control

As illustrated in the examples presented here, these zoonoses cross the human/livestock/wildlife interface. Because of this, effective surveillance and control strategies require a one health approach. These strategies should target different populations (human, livestock, wildlife) across the geography of the diseases. Central Asia and the Caucasus require improved livestock surveillance and vaccination strategies aimed at reducing the livestock burden of disease; this is true for both anthrax and brucellosis. Such strategies should have significant livestock and human health benefits. In contrast, the wildlife situation of anthrax in wild boar in Ukraine poses a different challenge. Anthrax vaccination is untenable in wildlife (11, 51). In the absence of vaccination, rapid carcass cleanup during outbreaks is the only apparent means of reducing the size of outbreaks (51). However, it is important that burial efforts result in deep burial to reduce potential for inadvertent digging that exposes carcasses, as bone can remain infectious for long periods of time. Because of this, there is a need to better understand the timing and spatial distribution of epizootics; such information comes from increased surveillance and environmental sampling. This would allow managers to stage preemptive surveillance and control efforts.

Ecological niche models (also referred to as species distribution models) can be used to broadly define the geographic range of *B. anthracis, Y. pestis,* and *F. tularensis* to better inform surveillance efforts. When coupled with spatial analyses of outbreaks, we can identify areas of high risk (where the clusters occur) and areas where passive surveillance should increase (where niche models predict in under investigated areas). Specific to anthrax, expanded surveillance and wildlife telemetry studies can assist in understanding the relationship between individual animals in a herd and their use of the landscape during anthrax risk periods (11). Such studies can shed light on the role of animal behavior in contacting the environmental reservoir for the pathogen. This could greatly improve our understanding of anthrax in Ukrainian boar populations.

Much of the recent spatial modeling of anthrax has relied on mortality data to understand the disease, which likely underestimates the extent and intensity of the disease (52). The data from boars suggest that pathogen exposure occurs beyond known foci, even without reported mortality events. Coupling data from across temporal and spatial scales and across host species in a modeling framework should provide better information on the disease that can be shared with wildlife managers, regional public health officers, and policy makers.

We have also illustrated that disease surveillance in these countries is anthropocentric and requires greater data sharing, cooperation, and funding to support joint human and veterinary surveillance and disease control. The work on anthrax in Azerbaijan highlights the role of livestock disease control for improving human health. Future efforts should expand this type of research to brucellosis studies across these countries. Regional approaches to zoonotic and transboundary diseases often require regional efforts in mitigation and vaccination strategies that are effective in reducing the propagation of the infectious diseases. Through the above collaborations discussed in this paper, the countries of Azerbaijan, Georgia, Kazakhstan, and Ukraine have developed a Regional Disease Surveillance Working Group (RDSWG) to foster communication and collaboration in disease surveillance of these pathogens. This is in direct response to the cooperative engagements with the countries and the results of research studies that show the importance of sharing of data and communication in reducing the impact of transboundary diseases.

The examples of plague and tularemia presented here illustrate the importance of continued small mammal and associated vector surveys across these countries. Each of these diseases is maintained in small mammal populations and is likely to maintain over long periods of time. Ultimately, surveillance is time-consuming and expensive and must be balanced against risk. In the work in Azerbaijan, Morris et al. (43) illustrated the use of high resolution spatial data mapping human populations can be compared to areas of historical disease foci to focus what are realistically limited surveillance dollars to those areas of greatest likelihood of human infection.

CONCLUSION

Across these examples, we have illustrated the value of cooperative studies that bring together modern geospatial and epidemiological analyses with historical and contemporary disease surveillance to improve our understanding of the distribution of pathogens and diseases in livestock, wildlife, and humans. The results of these efforts illustrated in this paper are all available as peer-reviewed studies. We advocate for the need to bring the results and the researchers from cooperative studies into meetings where policy is negotiated to best use these results to inform future disease surveillance and control or eradication campaigns. Each of the studies highlighted here identify local spatial heterogeneity in the distributions of these diseases. Such information should be considered critical for policymakers when considering strategies for reducing eradicating these diseases.

FUNDING

JB and IK were partially funded by the Defense Threat Reduction Agency through the Academic Engagement Program administered by Penn State University. Support for JB was also provided by the Emerging Pathogens Institute at the University of Florida.

REFERENCES

1. Frieden TR, Damon I, Bell BP, Kenyon T, Nichol S. Ebola 2014 – new challenges, new global response and responsibility. *N Engl J Med* (2014) **371**:1177–80. doi:10.1056/NEJMp1409903
2. Dixon MG, Schafer IJ. Ebola viral disease outbreak – West Africa, 2014. *MMWR Morb Mortal Wkly Rep* (2014) **63**:548–51.
3. Ansumana R, Bonwitt J, Stenger DA, Jacobsen KH. Ebola in Sierra Leone: a call for action. *Lancet* (2014) **384**:303. doi:10.1016/S0140-6736(14)61119-3
4. Gire SK, Goba A, Andersen KG, Sealfon RS, Park DJ, Kanneh L, et al. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. *Science* (2014) **345**:1369–72. doi:10.1126/science.1259657
5. Alexander KA, Lewis BL, Marathe M, Eubank S, Blackburn JK. Modeling of wildlife-associated zoonoses: applications and caveats. *Vector-Borne Zoonotic Dis* (2012) **12**:1005–18. doi:10.1089/vbz.2012.0987
6. Blackburn J. Integrating geographic information systems and ecological niche modeling into disease ecology: a case study of *Bacillus anthracis* in the United States and Mexico. In: Skowroski EW., O'Connell KP, editors. *Emerging and Endemic Pathogens: Advances in Surveillance, Detection, and Identification*. Dordrecht: Springer (2010). p. 59–88.
7. Hugh-Jones M, De Vos V. Anthrax and wildlife. *Rev Sci Tech* (2002) **21**:359–83.
8. Fasanella A, Galante D, Garofolo G, Jones MH. Anthrax undervalued zoonosis. *Vet Microbiol* (2010) **140**:318–31. doi:10.1016/j.vetmic.2009.08.016
9. Woods CW, Ospanov K, Myrzabekov A, Favorov M, Plikaytis B, Ashford DA. Risk factors for human anthrax among contacts of anthrax-infected livestock in Kazakhstan. *Am J Trop Med Hyg* (2004) **71**:48–52.
10. Blackburn JK, Hadfield TL, Curtis AJ, Hugh-Jones ME. Spatial and temporal patterns of anthrax in White-Tailed Deer, *Odocoileus virginianus*, and hematophagous flies in West Texas during the summertime anthrax risk period. *Ann Assoc Am Geogr* (2014) **104**:939–58. doi:10.1080/00045608.2014.914834
11. Blackburn JK, McNyset KM, Curtis A, Hugh-Jones ME. Modeling the geographic distribution of *Bacillus anthracis*, the causative agent of anthrax disease, for the contiguous United States using predictive ecologic niche modeling. *Am J Trop Med Hyg* (2007) **77**:1103–10.
12. Turner A, Galvin J, Rubira R, Miller G. Anthrax explodes in an Australian summer. *J Appl Microbiol* (1999) **87**:196–9. doi:10.1046/j.1365-2672.1999.00869.x
13. Parkinson R, Rajic A, Jenson C. Investigation of an anthrax outbreak in Alberta in 1999 using a geographic information system. *Can Vet J* (2003) **44**:315–8.
14. Blackburn JK, Goodin DG. Differentiation of springtime vegetation indices associated with summer anthrax epizootics in West Texas, USA deer. *J Wildl Dis* (2013) **49**:699–703. doi:10.7589/2012-10-253
15. Dragon D, Elkin B, Nishi J, Ellsworth T. A review of anthrax in Canada and implications for research on the disease in northern bison. *J Appl Microbiol* (1999) **87**:208–13. doi:10.1046/j.1365-2672.1999.00872.x
16. Fasanella A, Garofolo G, Hossain M, Shamsuddin M, Blackburn J, Hugh-Jones M. Bangladesh anthrax outbreaks are probably caused by contaminated livestock feed. *Epidemiol Infect* (2012) **1**:1–8. doi:10.1017/S0950268812001227
17. Hugh-Jones M. 97 global anthrax report. *J Appl Microbiol* (1999) **87**:189–91. doi:10.1046/j.1365-2672.1999.00867.x
18. Özkurt Z, Parlak M, Tastan R, Dinler U, Saglam YS, Ozyurek SF. Anthrax in Eastern Turkey, 1992–2004. *Emerg Infect Dis* (2005) **11**:1939. doi:10.3201/eid1112.050779
19. Kracalik IT, Malania L, Tsertsvadze N, Manvelyan J, Bakanidze L, Imnadze P, et al. Evidence of local persistence of human anthrax in the country of Georgia associated with environmental and anthropogenic factors. *PLoS Negl Trop Dis* (2013) **7**:e2388. doi:10.1371/journal.pntd.0002388
20. Kracalik I, Malania L, Tsertsvadze N, Manvelyan J, Bakanidze L, Imnadze P, et al. Human cutaneous anthrax, Georgia 2010–2012. *Emerg Infect Dis* (2014) **20**:261. doi:10.3201/eid2002.130522
21. Kracalik IT, Blackburn JK, Lukhnova L, Pazilov Y, Hugh-Jones ME, Aikimbayev A. Analysing the spatial patterns of livestock anthrax in Kazakhstan in relation to environmental factors: a comparison of local (Gi*) and morphology cluster statistics. *Geospat Health* (2012) **7**:111–26. doi:10.4081/gh.2012.110
22. Kracalik I, Abdullayev R, Asadov K, Ismayilova R, Baghirova M, Ustun N, et al. Changing patterns of human anthrax in Azerbaijan during the post-soviet and preemptive livestock vaccination eras. *PLoS Negl Trop Dis* (2014) **8**:e2985. doi:10.1371/journal.pntd.0002985
23. Cherkasskiy B. A national register of historic and contemporary anthrax foci. *J Appl Microbiol* (1999) **87**:192–5. doi:10.1046/j.1365-2672.1999.00868.x
24. Mullins J, Lukhnova L, Aikimbayev A, Pazilov Y, Van Ert M, Blackburn JK. Ecological niche modelling of the *Bacillus anthracis* A1. a sub-lineage in Kazakhstan. *BMC Ecol* (2011) **11**:32. doi:10.1186/1472-6785-11-32
25. Aikembayev AM, Lukhnova L, Temiraliyeva G, Meka-Mechenko T, Pazylov Y, Zakaryan S, et al. Historical distribution and molecular diversity of *Bacillus anthracis*, Kazakhstan. *Emerg Infect Dis* (2010) **16**:789–96. doi:10.3201/eid1605.091427
26. Joyner T, Lukhnova L, Pazilov Y, Temiralyeva G, Hugh-Jones M, Aikimbayev A, et al. Modeling the potential distribution of *Bacillus anthracis* under multiple climate change scenarios for Kazakhstan. *PLoS One* (2010) **5**:e9596. doi:10.1371/journal.pone.0009596
27. Kracalik I, Lukhnova L, Aikimbayev A, Pazilov Y, Temiralyeva G, Blackburn JK. Incorporating retrospective clustering into a prospective cusum methodology for anthrax: evaluating the effects of disease expectation. *Spat Spatio-temporal Epidemiol* (2011) **2**:11–21. doi:10.1016/j.sste.2010.06.001
28. Bezymennyi M, Bagamian KH, Barro A, Skrypnyk A, Skrypnyk V, Blackburn JK. Spatio-temporal patterns of livestock anthrax in Ukraine during the past century (1913–2012). *Appl Geogr* (2014) **54**:129–38. doi:10.1016/j.apgeog.2014.07.016
29. Blackburn JK, Skrypnyk A, Bagamian KH, Nikolich M, Bezymennyi M, Skrypnyk A. Anthrax in a backyard domestic dog in Ukraine: a case report. *Vector-Borne Zoonotic Dis* (2014) **14**(8): 615–7. doi:10.1089/vbz.2013.1519
30. Bagamian KH, Skrypnyk A, Rodina Y, Bezymennyi M, Nevolko O, Skrypnyk V, et al. Serological Anthrax Surveillance in wild boar (Sus scrofa) in Ukraine. *Vector-Borne Zoonotic Dis* (2014) **14**:618–20. doi:10.1089/vbz.2013.1521
31. Morgan E, Lundervold M, Medley G, Shaikenov B, Torgerson P, Milner-Gulland E. Assessing risks of disease transmission between wildlife and livestock: the *Saiga* antelope as a case study. *Biol Conserv* (2006) **131**:244–54. doi:10.1016/j.biocon.2006.04.012
32. Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: a re-emerging zoonosis. *Vet Microbiol* (2010) **140**:392–8. doi:10.1016/j.vetmic.2009.06.021
33. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. *Lancet Infect Dis* (2006) **6**:91–9. doi:10.1016/S1473-3099(06)70382-6
34. Roth F, Zinsstag J, Orkhon D, Chimed-Ochir G, Hutton G, Cosivi O, et al. Human health benefits from livestock vaccination for brucellosis: case study. *Bull World Health Organ* (2003) **81**:867–76.
35. Gurbanov S, Akhmedova S. Especially dangerous infections in Azerbaijan. In: Skowroski EW, O'Connell KP, editors. *Emerging and Endemic Pathogens: Advances in Surveillance, Detection, and Identification*. Dordrecht: Springer (2010). p. 39–43.
36. Abdullayev R, Kracalik I, Ismayilova R, Ustun N, Talibzade A, Blackburn J. Analyzing the spatial and temporal distribution of human brucellosis in Azerbaijan (1995-2009) using spatial and spatio-temporal statistics. *BMC Infect Dis* (2012) **12**:185. doi:10.1186/1471-2334-12-185
37. Sanodze L, Bautista CT, Garuchava N, Chubinidze S, Tsertsvadze E, Broladze M, et al. Expansion of brucellosis detection in the country of Georgia by screening household members of cases and neighboring community members. *BMC Public Health* (2015) **15**:459. doi:10.1186/s12889-015-1761-y
38. Mamisashvili E, Kracalik IT, Onashvili T, Kerdzevadze L, Goginashvili K, Tigilauri T, et al. Seroprevalence of brucellosis in livestock within three endemic regions of the country of Georgia. *Prev Vet Med* (2013) **110**:554–7. doi:10.1016/j.prevetmed.2012.12.005
39. Akhvlediani T, Clark DV, Chubabria G, Zenaishvili O, Hepburn MJ. The changing pattern of human brucellosis: clinical manifestations, epidemiology, and treatment outcomes over three decades in Georgia. *BMC Infect Dis* (2010) **10**:346. doi:10.1186/1471-2334-10-346
40. Gage KL, Kosoy MY. Natural history of plague: perspectives from more than a century of research*. *Annu Rev Entomol* (2005) **50**:505–28. doi:10.1146/annurev.ento.50.071803.130337
41. Gratz N. Rodent reservoirs and flea vectors of natural foci of plague. *Plague Man Epidemiol Distrib Surveill Control* (1999) 63–96.
42. Pollitzer R. *Plague*. Geneva: *WHO* (1954).
43. Morris LR, Blackburn JK, Talibzade A, Kracalik I, Ismaylova R, Abdullahyev R. Informing surveillance for the lowland plague focus in Azerbaijan using a historic dataset. *Appl Geogr* (2013) **45**:269–79. doi:10.1016/j.apgeog.2013.09.014

44. Esmaeili S, Azadmanesh K, Naddaf SR, Rajerison M, Carniel E, Mostafavi E. A serological survey of plague in animals in Western Iran. *Emerg. Infect. Dis* (2013) **19**(9):1549–51. doi:10.3201/eid1909.121829
45. Staples JE, Kubota KA, Chalcraft LG, Mead PS, Petersen JM. Epidemiologic and molecular analysis of human tularemia, United States, 1964–2004. *Emerg Infect Dis* (2006) **12**:1113. doi:10.3201/eid1207.051504
46. Charette JD. CDC Category explanation (A, B, C) overview. *Toxico-Terror Emerg Response Clin Approach Chem Biol Radiol Agents* (2007) **755**:329.
47. Hightower J, Kracalik IT, Vydayko N, Goodin D, Glass G, Blackburn JK. Historical distribution and host-vector diversity of *Francisella tularensis*, the causative agent of tularemia, in Ukraine. *Parasit Vectors* (2014) **7**:1–6. doi:10.1186/s13071-014-0453-2
48. Golkovskiĭ GM, Mitsevich GF, Khaĭtovich AB, Alekseev EV, Korchevskiĭ PG. Natural focus of tularemia on the Kerchen peninsula (Crimea). *Zh Mikrobiol Epidemiol Immunobiol* (1981) (10):99–101.
49. Sjöstedt A. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. *Ann N Y Acad Sci* (2007) **1105**(1):1–29.
50. Hightower J. *Examining the Distribution of Francisella Tularensis, the Causative Agent of Tularemia, in Ukraine Using Ecological Niche Modeling*. (2012).
51. Blackburn JK, Asher V, Stokke S, Hunter DL, Alexander KA. Dances with anthrax: Wolves (*Canis lupus*) kill anthrax bacteremic plains bison (bison bison bison) in Southwestern Montana. *J Wildl Dis* (2014) **50**:393–6. doi:10.7589/2013-08-204
52. Bagamian KH, Alexander KA, Hadfield TL, Blackburn JK. Ante-and post-mortem diagnostic techniques for anthrax: rethinking pathogen exposure and the geographic extent of the disease in wildlife. *J Wildl Dis* (2013) **49**:786–801. doi:10.7589/2013-05-126

Conflict of Interest Statement: Dr. Jeanne Fair is currently a program officer with the Defense Threat Reduction Agency. She was not involved with the studies reviewed in this paper, nor involved in this decisions to fund those projects. Jason K. Blackburn and Ian T. Kracalik have no conflict of interest to declare.

frontiers in Public Health

REVIEW
published: 26 November 2015
doi: 10.3389/fpubh.2015.00271

Building Infectious Disease Research Programs to Promote Security and Enhance Collaborations with Countries of the Former Soviet Union

James C. Bartholomew[1], Andrew D. Pearson[2], Nils Chr. Stenseth[3], James W. LeDuc[4], David L. Hirschberg[5] and Rita R. Colwell[2]*

[1] Lawrence Berkeley National Laboratory, University of California Berkeley, Berkeley, CA, USA, [2] Center for Bioinformatics and Computational Biology, University of Maryland Center for Advanced Computer Studies, University of Maryland, College Park, MD, USA, [3] Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, Oslo, Norway, [4] Galveston National Laboratory, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA, [5] Department of Interdisciplinary Arts and Sciences, University of Washington Tacoma, Washington, DC, USA

OPEN ACCESS

Edited by:
Hillary H. Carter, U.S. Department of State, USA

Reviewed by:
Paras Jain, Albert Einstein College of Medicine, USA
Li Xu, Cornell University, USA
Andrew Michael Hebbeler, U.S. Department of State, USA

***Correspondence:**
James C. Bartholomew
jcbartholomew@lbl.gov

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *22 June 2015*
Accepted: *12 November 2015*
Published: *26 November 2015*

Citation:
Bartholomew JC, Pearson AD, Stenseth NC, LeDuc JW, Hirschberg DL and Colwell RR (2015) Building Infectious Disease Research Programs to Promote Security and Enhance Collaborations with Countries of the Former Soviet Union. Front. Public Health 3:271. doi: 10.3389/fpubh.2015.00271

Addressing the threat of infectious diseases, whether natural, the results of a laboratory accident, or a deliberate act of bioterrorism, requires no corner of the world be ignored. The mobility of infectious agents and their rapid adaptability, whether to climate change or socioeconomic drivers or both, demand the science employed to understand these processes be advanced and tailored to a country or a region, but with a global vision. In many parts of the world, largely because of economic struggles, scientific capacity has not kept pace with the need to accomplish this goal and has left these regions and hence the world vulnerable to infectious disease outbreaks. To build scientific capability in a developing region requires cooperation and participation of experienced international scientists who understand the issues and are committed to educate the next generations of young investigators in the region. These efforts need to be coupled with the understanding and resolve of local governments and international agencies to promote an aggressive science agenda. International collaborative scientific investigation of infectious diseases not only adds significantly to scientific knowledge, but it promotes health security, international trust, and long-term economic benefit to the region involved. This premise is based on the observation that the most powerful human inspiration is that which brings peoples together to work on and solve important global challenges. The republics of the former Soviet Union provide a valuable case study for the need to rebuild scientific capacity as they are located at the crossroads where many of the world's great epidemics began. The scientific infrastructure and disease surveillance capabilities of the region suffered significant decline after the breakup of the Soviet Union. The U.S. Cooperative Threat Reduction (CTR) Program, a part of the U.S. Department of Defense, together with partner countries, have worked diligently to improve the capabilities in this region to guard against the potential future risk from especially dangerous pathogens. The dissolution of the Soviet Union left behind many scientists still working to study pathogens using antiquated protocols in unsafe

laboratories. To address this situation, the CTR program began improving laboratory infrastructure, establishing biosafety and biosecurity programs, and training scientists in modern techniques, with emphasis on biosurveillance and safe containment of especially dangerous pathogens. In the Republic of Georgia, this effort culminated in the construction of a modern containment laboratory, the Richard G. Lugar Center for Public Health Research in Tbilisi to house both isolated especially dangerous pathogens as well as the research to be conducted on these agents. The need now is to utilize and sustain the investment made by CTR by establishing strong public and animal health science programs in these facilities tailored to the needs of the region and the goals for which this investment was made. A similar effort is ongoing in other former Soviet Republics. Here, we provide the analysis and recommendations of an international panel of expert scientists appointed by the Cooperative Biological Engagement Program of the Defense Threat Reduction Agency to provide advice to the stakeholders on the scientific path for the future. The emphasis is on an implementation strategy for decision makers and scientists to consider providing a sustainable biological science program in support of the One Health initiative. Opportunities, potential barriers, and lessons learned while meeting the needs of the Republic of Georgia and the Caucasus region are discussed. It is hoped that this effort will serve as a model for similar scientific needs in not only the former Soviet Union republics but also other regions challenged by infectious diseases where the CTR program operates.

Keywords: microbial ecology, science education, infectious diseases, collaborative research, biosurveillance, global health security, epidemiology, microbial genetics

INTRODUCTION

Understanding complex factors associated with occurrence and spread of infectious diseases is of fundamental importance in promoting health security. The biological world is a complex and constantly changing landscape while significant progress has been made in identifying pathogens that cause disease and producing therapies and vaccines to counter them, the reality is that even the best efforts to mitigate a disease threat can be confounded. Lederberg commented on this phenomenon many years ago when he argued that humanity does not stand a chance against the power of microbes to develop resistance to therapies unless scientists can collaborate and combine their intellectual powers in research to understand and develop novel approaches to alleviate diseases (1). Improving the capabilities of developing regions of the world to understand and address outbreaks of infectious diseases is paramount to saving lives globally. It can add considerably to the intellectual reserve needed to address these problems and reduce the spread to other regions. While there are powerful research engines in Europe and the Western world, a thorough and deep understanding of the nature of an outbreak can often best be understood by research conducted as close to the site of the outbreak as possible. Unfortunately, much of the less well-developed world where most of the emerging infections arise lacks modern research capabilities that can provide the necessary understanding. And trying to understand environmental and cultural aspects of how an outbreak arises when working in a laboratory 3000 miles away from the outbreak is less effective and likely to take too much of the precious time needed to contain the threat.

While natural evolution of infectious agents and the emergence of new infectious diseases remain the major threat to health security, molecular engineering makes it possible to modify microorganisms with relatively few resources, and that raises the concern that terrorist groups will apply these technologies to achieve nefarious goals. Bioterrorism poses a genuine threat, especially in an unstable world. As terrorist activities increase, application of bioterrorism becomes more likely. This is a particular concern in the less developed world where the threat may be most serious. Just as it is difficult to predict exactly when the next natural infectious disease outbreak will occur, it is also difficult to predict how a bioterrorist will construct a biological weapon and where it will be used. An effective research program to address all these issues for a particular region must remain current with developments in the science of infectious diseases as well as knowledgeable about ecological pressures on the local microbial community. The common link in a program to deal with any kind of outbreak is to assemble the tools and the skills to rapidly understand the nature of the outbreak, the factors playing a role in its occurrence and spread, and therapies to counter the threat. To recognize a new outbreak, it is important to have a full understanding of the spectrum of infectious agents endemic in a region over time to provide a baseline to recognize any anomalies and for predicting future events. Such understanding requires effective biosurveillance coupled with research on those agents important for health security, both regionally and global.

The U.S. Cooperative Threat Reduction (CTR) Program has been active in many regions of the world to help reduce the threat from especially dangerous pathogens. This effort is particularly targeted to reduce the threat from biological weapons and began in Russia in 1997 following the publication of a report from the U.S. National Academy of Sciences on "Controlling Dangerous Pathogens: A Blueprint for U.S. – Russian Cooperation" (2) It is now active and effective in many of the former Soviet Union republics as well as many other countries around the world. Its progress is most advanced in the Republic of Georgia where it has built an extensive network of laboratories, trained many scientists, and promoted studies to understand the nature of pathogens endogenous to the region. The center piece of the CTR effort in the Republic of Georgia is the Richard G. Lugar Center for Public Health Research (CPHR) in Tbilisi, a modern well-equipped containment laboratory design to safely house especially dangerous pathogens and the research conducted on these agents. It was developed jointly by the U.S. and Georgian governments and constructed with funds allocated by the U.S. Department of Defense through the CTR program to provide an early warning system for occurrence and potential spread of infectious diseases originating in Georgia and impacting the rest of the world. Establishment of the new central laboratory and its associated satellite laboratories throughout the region represents a unique opportunity to provide state of the art advances in life sciences that will assist health professionals combat infectious disease pathogens of concern no matter what their origin. The system was designed to allow for studies to be conducted on identified pathogens with the utmost regard for biosafety of the workers and the community and to provide a rapid report of information to the rest of the world. With this system in place and operational, Georgia now has the laboratory system needed to meet reporting requirements under the WHO International Health Regulations (IHR) and the World Organization for Animal Health (OIE), as well as the capability to support the Global One Health Initiative.

The CPHR is not only an important resource for meeting the challenge of public and animal health improvement for Georgia and the Caucasus region but also has the potential to serve as a catalyst for science advances throughout the region. The CPHR has the potential for connecting Georgian science with the world community of scientists through a scientific program designed to support collaborations and build partnerships across the Georgian scientific community, the region, and internationally.

THE ADVISORY COMMITTEES

The Defense Threat Reduction Agency (DTRA) as part of the U.S. Department of Defense through CTR and its Cooperative Biological Engagement Program (CBEP) has been responsible for funding and managing much of the science that has been ongoing at the CPHR. As the construction of the CPHR was completed, CBEP recognized the need to establish an international committee of peers to provide advice and transparency for the scientific program at the CPHR and its satellite laboratories. An International Scientific Advisory Council (ISAC) was assembled in 2010 comprised largely of volunteer scientists from around the world. These scientists used their experience and training to analyze all aspects of the scientific strengths of Georgia's existing public and animal health programs and global partnerships. The ISAC developed and submitted to CBEP a set of overarching recommendations, outlining the steps needed to ensure a sustainable scientific program for the CPHR to meets the goals of Georgia and the region as well as the CTR Program.

International Science Advisory Council

The ISAC included internationally respected scientists each with a strong interest in promoting science in developing regions. **Table 1** lists members of the ISAC. While most of the ISAC had their scientific base in Europe or the U.S., their combined experience and countries of origin spanned the globe, from South America to China. It was important that Council membership included those who, while their science was pursued in

TABLE 1 | International Science Advisory Committee (ISAC).

Name	Expertise and affiliation[a]
James Bartholomew[b], Chairman	Microbial Genetics, Lawrence Berkeley National Laboratory, University of California Berkeley, Berkeley, CA, USA
Henry M. Blumberg	Clinical and Translational Research, Emory University, Atlanta, GA, USA
Rita R. Colwell[b]	Computational Biology, Bioinformatics, and Genomics, University of Maryland, College Park, MD, USA
Carlos Del Rio	Biosurveillance, Emory AIDS International Training Program, Emory University, Atlanta, Georgia, USA
Timothy P. Endy	Infectious Disease Division, Department of Medicine, Upstate Medical University, Syracuse, NY, USA
Jason Farlow	Academic Engagement Program, Pennsylvania State University, In country scientist, Tbilisi, Republic of Georgia
Adolfo Garcia-Saestre	Department of Microbiology, Department of Medicine, Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, NY, USA
Jeannette Guarner	Department of Pathology, Laboratory Medicine, Emory University, Atlanta, Georgia, USA
Tsotne Javahishvili	Department of Molecular Technologies, Ambrx Technologies, San Diego, California, USA
Paul Keim	Translational Genomics Research Institute, Northern Arizona University, Flagstaff, Arizona, USA
Teymuras Kurzchalia	Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany
James LeDuc[b]	Galveston National Laboratory & Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, USA
Andrew D. Pearson[b]	Epidemiologist, Computational Biology, Bioinformatics, and Genomics, University of Maryland, College Park, MD, USA
David Prangishvili	Laboratoire, Biologie Moléculaire du Gène chez les Extrêmophiles, Institut Pasteur, Paris, France
Bruno Sobral	Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, Virginia
Nils Chr. Stenseth[b]	Evolutionary Biologist, Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, Oslo, Norway
Adrian Whatmore	Department of Bacteriology, Animal and Plant Health Agency, New Haw, Addlestone, Surrey, UK
Ruifu Yang	Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, China

[a] *Affiliation at the time of serving on the ISAC.*
[b] *Also member of the Panel of Experts (POE).*

institutes located outside of Georgia, their roots and educational background were Georgian. This ensured that the Council would have the perspective of native borne Georgian scientists who were practicing their science internationally. All the scientists comprising the ISAC had considerable experience working with Georgian scientists so they already had developed mutual trust.

Panel of Experts

The recommendations put forth by the ISAC were high-level science and dealt primarily with many of the political steps needed to ensure a successful scientific program. To translate the ISAC recommendations into an action plan for the science, CBEP commissioned a Panel of Experts (POE) drawing on membership of ISAC and receiving the official sanction of the U.S. government. CBEP charged the POE to assess the ISAC recommendations and prepare a plan for integration and implementation of the recommended science at the CPHR. Drawing on the initial discussions of the ISAC, the POE was tasked to develop a Strategic Science Agenda (SSA) and an Implementation Plan (IP) to turn recommendations into actions to aid the Georgians utilize and sustain the resources of the CPHR and its associated laboratories.

The POE comprised James C. Bartholomew, Chairman; Rita R. Colwell, Andrew D. Pearson, Nils Chr. Stenseth, and James W. LeDuc. In 2015, David L. Hirschberg, Department of Interdisciplinary Arts and Sciences University of Washington Tacoma, joined the POE to aid in implementation of the plans for the Lugar Center. While the POE was a stand-alone committee, it used the full resources of the ISAC when necessary.

OPERATING PLAN OF THE ISAC AND POE

The ISAC developed its recommendations for implementing an effective science program on infectious diseases centered at the CPHR supporting the needs of the region, by first assessing existing programs that support the public and animal health. It also looked back at the Soviet programs, the approaches taken, and the results obtained to understand the history shaping the current programs. At the same time, the ISAC considered the rapidly expanding understanding of the complexity of the biological world brought about by modern analytical techniques and how these advances could be applied to address issues unique and important to the region. It reviewed current plans for operating the CPHR and the laboratory network in Georgia, including long-term funding possibilities, staffing, training, logistics, and other resources. It met with long time collaborators on scientific project in Georgia from the U.S. Department of Defense Laboratories, the U.S. CDC, and Universities as well as scientists from international agencies with experience in Georgia to get their perspective on what was need to build a meaningful sustainable scientific program. It also discussed the issues with the U.S. Civilian Research and Development Foundation (CRDF Global) and the Shota Rustaveli National Science Foundation. Both of these organizations have extensive histories working together to support science in Georgia. These analyses were necessary to establish the base from which an appropriate future scientific program could be developed.

The most important part of the operating procedure of the ISAC was to hold extensive meetings with Georgian scientists to understand their perspective of the tasks need to develop a competitive program in health-related sciences. As part of this process, the ISAC worked with the management of the CPHR, the National Center for Disease Control and Public Health (NCDC&PH), the George Eliava Institute for Bacteriophage, Microbiology and Virology, and the Laboratory of the Ministry of Agriculture to develop a symposium entitled "Integrating Science and Public Health in Georgia." The symposium was designed to give young Georgian investigators the opportunity to discuss their work with the ISAC and to stimulate open discussion on the current state of research on infectious diseases in the region. The symposium was held at the auditorium of NCDC&PH on March 11, 2011, and was attended by over 100 scientists from throughout the region, including representatives from the Georgian Ministries, WHO, U.S. CDC, U.S. DoD, USAID, and the U.S. Embassy. Many of the current international collaborators on Georgian research projects also were in attendance, as well as CBEP science team members. The science discussed was not only the work supported through the DTRA CBEP but also the relevant work supported by other U.S. agencies and international funding groups.

The symposium gave the ISAC the opportunity to discuss with the Georgian scientists the goals of its effort, learn from the Georgians the issues that they felt needed to be addressed, and explore solutions. After the symposium, the ISAC continued its discussions of these issues via teleconferences and email, producing a final set of overall recommendations which it submitted to the CBEP science team. These recommendations addressed not only the science that needed to be promoted but also issues dealing with how the management of the program needed to be tailored to nurture the scientific program into the future. It challenged both DTRA and the Georgian Ministries to rethink their approach to managing the activities at the CPHR and to provide the necessary resources to establish and internationally recognized science program for the region. The recommendations were applicable not only for the immediate future but also for long-term maintenance of the health security of the region. The ISAC emphasized that the science to be conducted must address infectious disease problems relevant to Georgia and the region or the program would be overshadowed by similar programs in other parts of the world. The portfolio of science recommended by the committee provided for the establishment of a wide variety of skill sets for the Georgians to deal effectively with emerging infectious diseases, whether natural, the results of laboratory accident, or a deliberate act of bioterrorism. At the same time, the projects that were selected were to address the specific infectious disease issues relevant to the Caucasus region.

The ISAC also took into account the stakeholders of the health security system that had been established in Georgia and the Caucasus region. While the Government of Georgia was the lead stakeholder, the main financial stakeholder was the United States Department of Defense through DTRA and CTR. While this investment was significant, it was not likely to continue throughout long into the future. CBEP operates in Georgia with a defined mission to counter weapons of mass destruction through

modernizing the detection, reporting, and containment capabilities of the region. The ISAC concluded that for this mission to remain a success into the future it needed to be coupled with a program that had tangible benefit to the public and animal health of the region as recognized by the local governments.

International Scientific Advisory Council also recommended the CPHR establish formal relationships with international reference laboratories and scientists within international research centers conducting studies of relevance to the mission of the CPHR. Such collaborative relationships would aid in linking the CPHR program with international partners and integrate findings of studies undertaken at the CPHR into those conducted around the world. To provide an economic driver, the ISAC recommended that CPHR research activities be connected to an effort to incubate new biotechnology enterprises in Georgia and be colocated with a Biotechnology Center or "Farm." This would be spearheaded by a focus group composed of international biotechnology industry experts and provide long-term career possibilities for Georgian scientists as well a manufacturing site for products to be delivered to Georgian and regional markets.

Central to the ISAC recommendation is that core funding be available to allow for implementation of the joint programs that were recommended and to establish competence to conduct studies of relevance to international and national funding sources. The CPHR management will need to build communications with international funding agencies early so that, as the program matures, the accomplishments of the Georgian program would be recognized by the world community of scientists. This effort can be aided greatly with the help of internationally recognized scientist such as those that make up the ISAC and POE.

DEVELOPMENT OF A STRATEGIC SCIENCE AGENDA AND IMPLEMENTATION PLAN

The work to translate the recommendations of the ISAC into actionable projects to be conducted at the CPHR was the responsibility of the POE. The POE continued the discussions initiated by the ISAC and worked closely with the Georgian scientists to develop specific recommendations for the details of the science needed in the program. The effort included frequent trips to Georgia, as well as "kitchen table" discussions in Washington, DC, USA, teleconferences, and email. The team also realized that a complete understanding of the Georgian scientific situation required more than discussions across a meeting table or through a computer screen, and so it worked side-by-side with Georgian scientists to develop ideas including accompanying them on field trips for sample collection at sites within Georgia. The POE continuously encouraged the Georgian scientists to identify questions they believed needed to be addressed and the hypotheses they had developed to solve important infectious disease issues in the region. Throughout the process, the POE's approach to formulating their recommendations was to listen closely to the Georgian scientists, observe, and learn what the Georgian scientists needed to bring about a successful science program. The IP developed by the POE was a direct result of integrating all these ideas and coupling them to the POE's own experiences through the years in building and maintaining scientific programs at their home institutions.

The POE recognized that a scientific program that was not focused on issues important for Georgia and the region would soon be judged irrelevant and unsustainable. Central to this blueprint was a science plan that provided attainable goals that would build research momentum for Georgian scientists while providing important new information to the world's scientific community. Important were recommendations to energize young Georgians to pursue science at home to improve the health and welfare of the region. Young Georgian scientists must be encouraged to undertake the training and achieve the confidence needed to compete globally in international science at the very highest level by initiating international collaborations consonant with the rapid developments that are occurring in the biological sciences today.

The SSA was developed to emulate the concept of One Health Initiative (3) for the region by developing research projects that bring animal health professionals and human health professionals to the same laboratory working on collaborative projects on the study of ecological factors playing a role in pathogen evolution. It also strongly supports the need for an integrated multidisciplinary approach to solving infectious disease questions. As such, it builds off the existing programs at the CPHR, but takes them to the next level where they can answer truly new questions about infectious diseases while it focuses on those uniquely important factors of relevance to Georgia and the region. This includes knowledge of the ecology of disease causing microorganisms with the fundamental goal to inform all health professionals in the region.

STRATEGIC SCIENCE AGENDA

The POE recognized that the science proposed in the SSA had to address significant problems identified by surveillance data that were already available in the region and by CPHR research that was being supported by CBEP. The SSA needed to address the health needs of the region as a first priority with a base broad enough to allow it to address more global issues. Furthermore, the SSA called for enhancing the link between biosurveillance and fundamental research so as to take the studies of microbial ecology and evolution to a new level and be a model for similar studies in other regions. Skills developed in these studies would allow for rapid application to any future outbreak regardless of its source. Also clear was that all activities must be conducted following internationally approved protocols, and these protocols must be appropriate for agents in the environment of Georgia. Long-term sustainability is dependent on recognition of the importance of problems addressed, quality of data collected, and relevance both to the region and the world.

The POE called for the investigators conducting work at the CPHR to address the complexity of the microbial world and to utilize assays relevant to the problems of concern. While classical phenotypic-based assays have limitations with respect to sensitivity, accuracy, and safety, their results are still considered meaningful (4); molecular assays are highly discriminatory but must be validated and shown to be clearly related to the

pathogenic phenotype (5). The array of markers that define pathogenicity is complex and not completely understood, but it is clear that any one marker cannot determine biological activity of the agent. The SSA calls for selecting appropriate assays based on a thorough knowledge of the pathogens relevant to the local situation.

The fundamental element in the SSA that focused all the proposed science is the proposal for the Caucacus Microbial Ecology Project (CMEP; **Figure 1**), studies to understand the complex microbial ecology and evolution related to pathogens of concern to Georgia and the region. CMEP would leverage off many of the existing science projects being conducted at the CPHR supported by CBEP, including the nucleic acid sequencing and annotation efforts, the Geographic Information System (GIS) mapping of pathogens, and strain characterization efforts, but would add a complex multifactorial aspect to the work to address the ecological parameters that shape changes to infectious agents. New skills would be added to the program at the CPHR including a more refined analysis of data, hypothesis generation, and the creative design of approaches to answer important question. The scientists of the CPHR would be encouraged to develop high-resolution studies to map the baseline occurrence of pathogens in the environment related to the occurrence of disease in human beings and animals. Studies were proposed to determine how complex microflora can affect activities of specific pathogens and their potential interactions with and in their host, reservoir, and vector. Recognizing that the environment represents a very extensive microbial "reservoir," the POE recommended developing and utilizing assays to distinguish pseudo-pathogens and those relevant to diseases in the region. The ultimate goal is to provide a risk analysis of exposure to pathogens, as well as recommendations for a mitigation strategy. Of particular concern was to build projects that strengthen the working relationship between scientists studying zoonotic infectious diseases. It was clear that without a strong partnership between animal health experts and human health experts, the One Health Initiative would fail. The knowledge gained from these studies would allow understanding the pathogen spectrum of the region, namely what is there now and how, or if, it is changing, so that introduction of a new agent could be recognized. Understanding the situation in the Caucasus region would provide information that could be compared and integrated into similar studies conducted in other parts of the world, thereby achieving a global perspective.

Linking Biosurveillance and Fundamental Research

The SSA contained several recommendations to assist the development of a biosurveillance program informed by a regional specific research agenda.

- Develop high-resolution epidemiological typing systems for regionally relevant pathogens that is based on internationally recognized methods that allow comparison of local data with those of global isolates.
- Devise schema to improve coordination and resources utilization of existing infectious disease programs sponsored by Georgia and/or international agencies. This will provide a basis for a "One Health Initiative" in Georgia.
- Establish a national plan to improve laboratory diagnostics for the region, notably strengthen coordination of surveillance for human and animal infectious disease agents that pose public and animal health problems.
- Develop an overall integrated disease reporting system for Georgia applicable to region-significant infectious diseases that is based on syndromic surveillance and coupled to the enhanced laboratory capacity of the CPHR.
- Improve diagnostic capabilities based on biological activities relevant to pathogenicity and baseline disease knowledge.

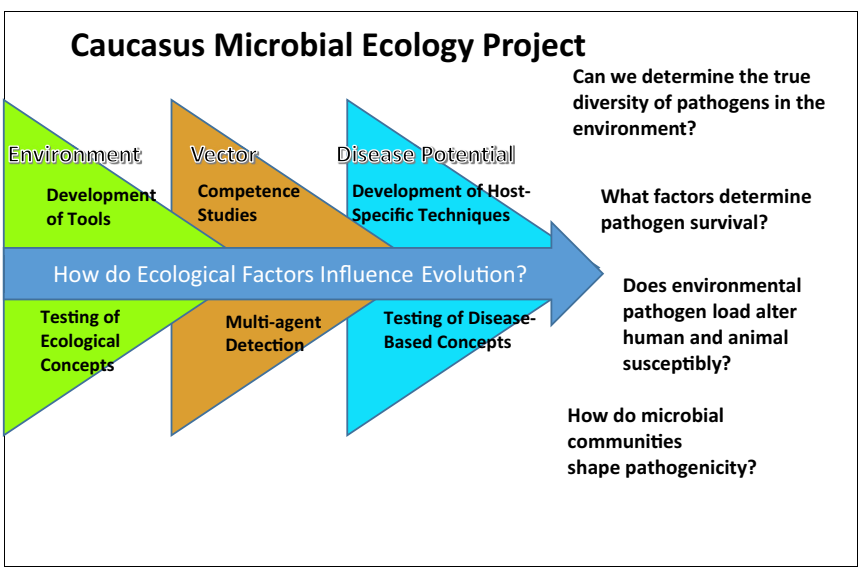

FIGURE 1 | Microbial ecology and evolution of pathogens in the Caucasus – Study Plan.

This will promote implementation of relevant disease control strategies as appropriate to the region.
- Include surveillance data on pathogen near relatives to assess impact on host susceptibility.
- Establish a well-documented sample and data repository to allow reach back to samples for historical analysis, based on current understanding of relevant problems.
- Develop a knowledge-based risk benefit analysis to deal with infectious disease issues and allow efficient application of resources.
- Study the interplay of different geographical factors and how they influencing agent distribution. For example, the geographical distribution of the use of antibiotics and the appearance of resistant microbes.
- Understand the relationship of the landscape distribution between different agents.
- Determine the distribution of susceptible populations and vectors, and the factors controlling susceptibility.
- Apply statistical modeling of historical data and relevant factors determined to be functioning in the current environment.
- Study the factors influencing clonal distribution of pathogens in the environment.
- Study of genetic varieties of a given pathogen or multiple pathogens and whether they exclude one another or enhance occurrence in the region? Determine the factors that drive these relationships.
- Study of near relatives of pathogens and their role in stimulating natural immunity.
- Study the role of immunological enhancement of one version of a pathogen on another to modulate activity of pathogens of importance to the region.

Topic Areas for Fundamental Research

The SSA called out specific fundamental research areas to be contained in the CMEP. These topic areas were select based not only on their relevance to the focus of microbial ecology but also because the skills developed during studies in these areas would transcend many other research projects of relevance to the mission of the CPHR. Studies in these areas would add capabilities to existing expertise in strain characterization and genomics being developed by CBEP at the laboratory. Another concern in selecting these topic areas was to provide opportunities to develop projects that supported and involved scientists from both the public health sectors as well as animal health scientists with a bridge to the phage research at the Eliava Institute, which is a unique and strong program in Georgia.

1. Antibiotic-resistant pathogens – detection and genomic characterization of multiple drug-resistant agents; auditing national antibiotic usage in people, animals, and agriculture; analysis of outcomes of antibiotic usage correlated with bacterial resistance patterns.
2. Vector-borne diseases – epidemiological analysis of human and environmental pathogens linked with vector-borne diseases, notably exploration of hypotheses for the molecular basis of environmental maintenance of disease agent foci in Georgia, including infections with multiple pathogens and investigation of prevalence and impact of multiple pathogens transmitted by single vectors.
3. Pathogen migration – molecular epidemiology of avian influenza linked to animal/avian borne human cases to address evolution of sequences of these viruses in the region and relationship to influenza sequences of viruses globally.
4. Zoonotic risk assessment – impact of zoonotic brucellosis on human health and veterinary practice: assessment of health gain from potential prevention strategies.
5. Food and water-borne pathogens – impact of zoonotic salmonellosis on human health and veterinary practice.
6. Horizontal gene transfer – nucleotide sequence and annotation of bacteriophages in the Eliava collection, with emphasis on horizontal transfer of genes in the evolution of pathogens.

These topics circumscribe important capabilities either already present or needed at the CPHR and associated satellite laboratories to support public and animal health in a twenty-first century research facility. The portfolio of topics and the derived projects will build relevant technical capabilities and practical confidence in Georgian science. To ensure successful implementation of the plan, scientific partners would be sought to assist in applying lessons learned from published scientific research in international settings that was carried out in programs that have relevance for Georgia. This will provide Georgian scientists with an opportunity to learn new methods, refine scientific skills in an international arena, and obtain relevant information for their research, as well as inform decision makers in Georgian Government agencies.

The SSA was submitted to the management of the Lugar Center and the CBEP science team in November, 2012, and was approved.

THE IMPLEMENTATION PLAN

Cooperative Biological Engagement Program and the POE realized that for the SSA to be useful, it needed an effective IP to serve as a catalyst for action. The POE relied heavily on its own experience with what it takes to carry out successful completive research to develop the IP. It takes a critical mass of discussion amongst knowledge scientists addressing similar problems to push research in new directions. It takes a vision to focus the research and provide a goal to be achieved. Coupled with this is that it takes persistence in the effort to obtain funding, as the road to receiving the funding is filled with many setbacks. These setbacks need to be used as lessons learned to improve proposals for another try. It takes support from other scientists both locally and internationally as well as support from home institutes and governments who understand that quality science requires a period of incubation.

Figure 2 outlines the key steps in the IP. The plan is made up of steps to stimulate research ideas, plans to train in the unique aspects of managing research projects, and steps to assist in developing long-term funding to support the work of the SSA. Along the way new contacts are made with recognized scientists in areas relevant to program in Georgia.

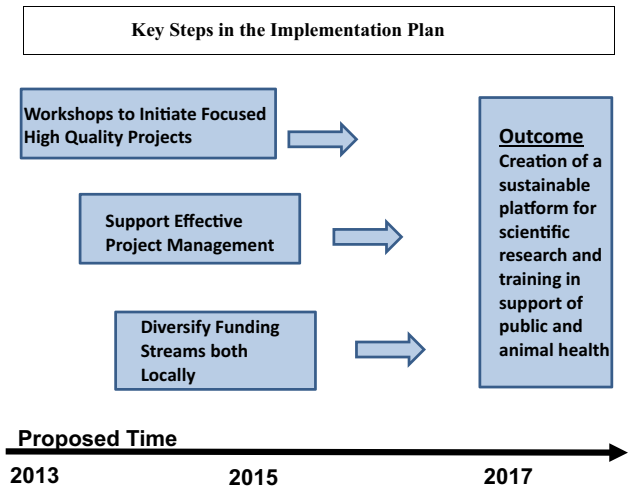

FIGURE 2 | Steps in the implementation plan. The raw material for a successful science agenda is a set of high-quality research projects that excite both investigators and the scientific community that is strongly supported by institute management.

As called out in the IP, the POE is to conduct scientific workshops on topic areas called out in the SSA. The goal is to bring a number of highly respected international scientists to Tbilisi to present results from their own studies conducted in their laboratories. The idea was to stimulate discussions with the Georgian scientists on "state of the art" research and how it might fit into future work at the CPHR. The workshops were to be hosted by Tbilisi State Medical University (TSMU) and by NCDC&PH and be designed to allow for plenty of discussion. It was through these workshops that the POE hoped to generate new ideas to serve as the focus of new proposals from the Georgian scientists in collaboration with international partners. It was hoped this would be an educational experience for both sides – the Georgian scientists would hear the work ongoing in their fields in international laboratories, and the invited scientists would see the possibilities of collaborative work in Georgia and the region.

Workshops Progress

The POE, with the help of the Georgian Institutes, the U.S. Embassy in Georgia, and contractors for the CBEP began organizing workshops focused on the six research topic areas listed in the SSA. The workshops included Georgian scientists and international experts, with a sharing of experience and lessons learned with specific hypotheses to be tested. Workshops conducted to date have led to development of 18 competitive proposals for projects. Each workshop has included productive discussion leading to establishment of objectives for each topic area and appropriate hypotheses to be tested that suit the needs of the Georgian public and animal health objectives.

Two workshops have been held and jointly hosted by TSMU and the NCDC&PH. Both workshops were opened by the United States Ambassador to Georgia, the Rector of the University, and the Director of NCDC&PH.

The first workshop, International Workshop on Antimicrobial Resistance (AMR), was held on July 2–4, 2013. More than 200 regional scientists and scientists from countries of the former Soviet Union attended. At this first workshop, Memoranda of Understanding (MOU) to committing parties to work together to develop biological sciences in Georgia were signed by officials of TSMU, NCDC&PH, and POE members representing the University of Maryland, United States, and the University of Oslo, Norway.

As shown in **Table 2**, speakers were from many different countries representing different perspectives, but all focused on

TABLE 2 | International Workshop on Antimicrobial Resistance (AMR).

Workshop #1

Speaker	Affiliation	Title
Fred Tenover	Stanford University and Cepheid, Palo Alto, CA, USA	Use of Appropriate Technologies for the Rapid Diagnosis and Surveillance of Antimicrobial Resistance in Georgia and the Caucasus
Bruce R. Levin	Emory University, Atlanta, GA, USA	Role of Microbial Pathogen Population Dynamics in the Spread of Antimicrobial Resistance
Martin F. Polz	Massachusetts Institute of Technology, Cambridge, MA, USA	Ecological Populations of Bacteria Act as Socially Cohesive Units of Antibiotic Production and Resistance
Nadezhda Fursova	State Research Center for Applied Microbiology and Biotechnology, Obolensk, Russia	The Novel CTX-M-116 β-lactamase Gene Discovered in Proteus mirabilis is Composed of Parts of the CTX-M-22 and CTX-M-23 Genes
Tomi Kostyanev	Laboratory of Medical Microbiology, University of Antwerp, Belgium	European AMR COMBACT LAB Network
George Kamkamidze and Nino Macharashvili	Richard G. Lugar Center for Public Health Research, Tbilisi, Republic of Georgia	Gram Negative Infections in Hospital and Community Patients
Mikeljon Nikolich	WRAIR, WHO & U.S.A. CDC	AMR Surveillance in Georgia – summary of current and proposed activity: scope of the project team proposal for surveillance and research
Giorgi Chakhunashvili	National Center for Disease Control & Public Health, Tbilisi, Republic of Georgia	AMR in Georgia
Ekaterine Zangaladze	National Center for Disease Control & Public Health, Tbilisi, Republic of Georgia	MDR and XDR TB infection surveillance and control in Georgia
Rezo Adamia	George Eliava Institute of Bacteriophage, Microbiology and Virology, Tbilisi, Republic of Georgia	Bacteriophages as Potential New Therapeutics to Replace or Supplement Antibiotics
Rita Colwell	University of Maryland Center for Bioinformatics and Computational Biology, College Park, MD, USA	Steps to move forward to address AMR from an ecological and molecular genetic perspective

solving local AMR problems in Georgia as part of a global effort. The POE designed the workshops to bring together scientists from countries outside the former Soviet Union and to reunite scientists from within the former Soviet Union to stimulate a transparent environment for research, especially at the CPHR.

From this workshop, Georgian scientists and international experts developed and proposed a set of 18 competitive proposals with Georgian investigators as Principle Investigators. The POE reviewed 18 proposals and selected the top five, recommending these to CBEP for their consideration for funding through DTRA's Basic and Fundamental Research Broad Area Announcement. The CBEP science team and reviewers considered all five of the proposals and selected the one which described a regional effort to assess occurrence of carbapenem-resistant bacteria circulating in the region for funding and recommended the proposal to DoD Policy for approval. Unfortunately, DoD Policy ruled that the work described in the proposal was outside the scope of the CBEP mission and the proposal was not funded.

While this workshop did not result in proposals being funded by DTRA, it did generate excellent proposals that receive excellent scientific reviews. These proposals will be submitted to other funding agencies whose focus is on the world-wide crisis of AMR. And it provided Georgian investigators very useful experience in preparing competitive proposals to attract international funding for their research. It also emphasized and called attention to the need to develop a program for the region to counter the threat of antibiotic-resistant strains of pathogens arising from unregulated use of antibiotics that has been the practice in this region for decades.

The second POE workshop (**Table 3**), Microbial Ecology of Environmental Pathogens (MEEPs), was held on December 4–6, 2014, at the same venue as the first workshop. The attendance was similar to that of the workshop on AMR. Again, discussion was lively and generated many new ideas. The workshop objective was to understand how microorganisms interact with their environment, with each other, with their vectors, and with their hosts. There was an emphasis on how ecological factors of Georgia and the region shape development of new strains and/or species of environmental pathogens and how development of newly emerging infections occurs. The program highlighted presentations of both Georgian scientists and international invitees, with excellent sharing of experiences and lessons learned from molecular-based investigations into scientific question(s) surrounding health security. This workshop, as did the first workshop, focused on

TABLE 3 | Microbial Ecology of Environmental Pathogens (MEEPs).

Workshop #2

Speaker	Affiliation	Title
Michael J. Mahan	University of California Santa Barbara, Santa Barbara, CA, USA	Rise of the Microbes
Elisabeth Carniel	Institut Pasteur, Paris, France	Horizontal Acquisition of a Filamentous Phage Early after Y. pestis Emergence
A. Marm Kilpatrick	University of California, Santa Cruz, CA, USA	Drivers, Dynamics and Control of Emerging Vector-borne Zoonotic Diseases
Peter Hudson FRS	The Huck Institute of Life Sciences, Penn State University, PA, USA	An Ecological Perspective on Spillover and Invasion of Infectious Diseases
Gvantsa Chanturia	National Centre for Disease Control and Public Health, Tbilisi, Republic of Georgia	Review of Tularemia Ecology in Georgia
Ekaterine Khmaladze	National Centre for Disease Control and Public Health, Tbilisi, Republic of Georgia	Discovery and Further Investigation of a New Highly Divergent Orthopoxvirus in Georgia
Giorgi Babuadze	National Centre for Disease Control and Public Health, Tbilisi, Republic of Georgia	Detection, Confirmation and Phylogenetic Analysis of Crimean-Congo Hemorrhagic Fever Virus in Human and Tick Samples Obtained During 2013-2014 Outbreaks in Georgia
Anna Machabilishvili	National Centre for Disease Control & Public Health, Tbilisi, Republic of Georgia	Transmission of Zoonotic Influenza between Humans, Pigs, and Poultry
Robert Webster	Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA	Perspectives on Influenza Evolution and the Role of Research
Kornelia Smalla	Julius Kühn-Institut-Federal Research Centre for Cultivated Plants, Braunschweig, Germany	Genes in Motion – Widespread dissemination of class I integron components in soils and related ecosystems as revealed by cultivation-independent analysis
Jason Farlow	Farlow Scientific Consulting, Lewiston, UT, USA	Ecological and Within-host Implications of Viral Quasispecies
David Prangishvili	Institut Pasteur, Paris, France	Viruses of the Archaea: insights into the diversity and evolution of virus-host interactions
Marina Tediashvili and Ekaterina Jaiani	George Eliava Institute of Bacteriophages, Microbiology and Virology Tbilisi, Republic of Georgia	Diversity and Predictability of Human Pathogenic Vibrios along the Georgian Coastal Zone of the Black Sea
Britt Koskella	University of Exeter, Cornwall Campus, Tremough, TR10 9EZ, UK	Understanding Bacteriophage Specificity in Natural Microbial Communities
Marina Donduashvili	Laboratory of the Ministry of Agriculture, Tbilisi, Republic of Georgia	Epidemiological and Laboratory Surveillance of CCHF in Animals in 2014
Dennis Bente	Galveston National Laboratory, University of Texas Medical Branch, Galveston, TX, USA	Pathogenesis and Transmission of Crimean-Congo hemorrhagic fever virus
Yingzi Cong	University of Texas Medical Branch, Galveston, TX, USA	The Dynamic Influence of Commensal Bacteria on the Immune Response to Pathogens

identifying competitive research projects reflecting the needs of Georgian science to support public and animal health. The discussions resulted in the establishment of a set of objectives for each topic that had been identified and scientific hypotheses addressing those topics. An assessment of relevant technology and expertise to deliver project goals relevant to Georgian was provided. The international team continues to work on a broad range of topics with the Georgian scientists on how the environment serves as a natural reservoir of pathogens and how microorganisms alter their genetic composition to counter threats to their survival induced by ecological pressures caused by human activity. It is intended that the outcomes of this workshop will include proposals to study ecological drivers of pathogen change and identify new tools and new approaches to mitigate risk of emerging infections and reduce the burden to public and animal health.

A request for proposals (RFP) derived from the second workshop has not yet been issued. However, the participants continue to engage in discussions of ideas to be developed into projects to propose when the RFP is released.

The POE continues to develop plans for additional workshops to support the SSA. While a proposal has yet to be funded, the value of the program is abundantly clear. Georgian scientists have had experience in developing their own ideas for competitive research proposals. Many of them are committed to follow through even though they have not yet been successful in receiving funds from DTRA to carry out the work. However, through the process, they have built relationships with many new scientists from around the world and the discussions that followed have generated new perspectives on ideas of how to attack the problems of infectious disease in their region. The work on these projects needs to continue to fully develop these ideas as the Georgian and regional program matures.

Focus Groups

Success in implementing the SSA would require effective communication fostering creative hypothesis-based research. Building a communication network not only within a project team but also throughout the institution was required to share information and ideas across project boundaries to leverage expertise throughout the program. Therefore, POE proposed the focus group concept to explore core needs of the science program. The focus group concept is based on realization that a critical mass of scientists is needed to build a sustainable program. In a complex multidisciplinary program, maintaining expertise in a technical area is often sacrificed by the team's focus on the goal. Focus groups are designed to cut across project boundaries and allow specialists to maintain their expertise while contributing to many projects and to the overall program. A focus group comprises those individuals engaged in projects in Georgia, including international infectious disease programs and investigations. A group of experts is assembled, representing disciplines needed for advising projects underway at the CPHR. This will maximize support and integration of disciplines. Microbial ecology research is best done with an interdisciplinary approach, and interdisciplinary science works best when each discipline is fostered to a high level of expertise, while communicating lessons learned across project boundaries.

The focus groups would be composed of both Georgian and international scientists working as collaborators from their home institutions, or on site at the CPHR. The goal of the focus groups was to ensure the best possible approach was taken for each of the implemented projects to be achieved and that the results and ideas were shared to maximize efficiency and progress of the program as a whole. While the design of the focus groups is flexible to meet the needs of the specifics in the program, one possible starting point is illustrated in **Figure 3** where the focus groups comprise pillars that link research with the public and animal health agencies providing a two way conduit of information and ideas.

Scientific Project Management

Successful programs in scientific research depend on translating ideas into scientific results. To foster successful projects at the CPHR implementation of effective scientific management dedicated to the highest quality science is mandatory. While the front line of a management team is the Georgian scientist team trained in scientific project management, during the initial phase of implementation of a SSA, support of an international team of collaborating scientists (with experience in project management) is crucial.

Diversifying Scientific Funding

While developing the IP, the POE acknowledged that funding for science is highly competitive and funds are very limited, especially for programs where the Principal Investigator scientists are just beginning to establish their reputations. Thus, the POE will be assisting CPHR management in obtaining funding needed to conduct research outlined in the Strategic Scientific Plan. New ideas, resources, experience, and a nurturing scientific environment are all critical to success. While the experience of entering the competitive fray has much to offer in lessons learned and partnerships made, it can be frustrating. A new facility, like the CPHR, makes it necessary to procure funds for operational needs and seeking funds for the research to be done becomes even more challenging. Fortunately, there are international programs that support international scientific development. The POE is to work with the CPHR and international partners to target the relevant funding agencies.

Building Blocks for Program Development

The POE continues its work with the Georgian scientists and policy makers to develop a strong program of scientific research. Criteria for success will be the establishment of a portfolio of projects undertaken at the CPHR supporting the overall program. The scientific projects will add value to the CPHR program and aid in development of new initiatives for the Georgian Universities. Tools to be included in the program are quantitative bacteriology, phylogenetic analysis, molecular cloning, quorum sensing, informed biosurveillance, and molecular epidemiology, among others. All serve as a basis for the Focus Groups. The goal is to assemble, from the start, projects where the whole is greater than the sum of the parts. It is important to point out that these tools are building blocks and not what are usually considered to

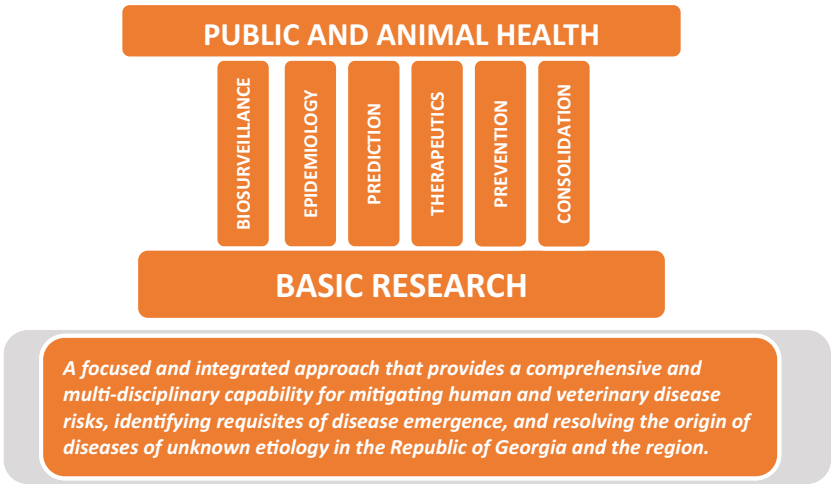

FIGURE 3 | Focus groups. The POE Implementation Plan envisioned Focus Groups as pillars to ensure strong linkage between the needs of the public and animal health identified issues and the basic research program conducted at the CPHR enhancing the ability of the health sector to respond to disease outbreaks and providing relevance to the research program.

be the tools of science, such as an electron microscope or nucleic acid sequencer, but instead people oriented capabilities that can be utilized to understand a general biological process central to a larger question. These building blocks will be important to the regional scientists in understanding disease outbreaks no matter whether its origins are natural, from laboratory accidents, or from intentional releases of synthetic pathogens.

The IP is intended to serve ongoing efforts and capitalize on unique resources and talents in Georgia to provide support for the One Health Initiative.

In summary, the POE provides a unique resource in assistance and guidance to CPHR management as the struggle for diversification in science research and expansion of their funding base get underway. The extended network of international scientists of the POE offers a useful and flexible means for identifying new sources of funding. The POE can work closely with CPHR management to identify funding sources and assist in procuring funding. International funding partners need to be identified and selected scientific areas of research underway should be showcased to leverage the unique resources of CPHR, Georgia, and the region. The POE members can assist in developing proposals and by communicating the progress being made at the CPHR and ensuring CPHR management is informed of scientific funding trends.

LESSONS LEARNED AND CONCLUSION

The efforts described here do not represent the first group of experienced senior scientists to commit themselves to helping build scientific programs to improve public and animal health in developing regions (6). While the location and details may be different with each such effort, the lessons learned are more often similar. The scientific questions are not what presents the greatest challenge. Nor is it the availability of modern laboratories and scientific equipment that determines success. The most difficult challenge is to obtain the commitment to build the educational foundation of a strong scientific program and to stay the course for the long term, through the frustrating times of establishing a world-class science program. Building a science program in a developing region requires governments and international funding agencies to work together and to understand that seed funds are critical to support the effort. In Georgia, the CRDF Global and the Shota Rustaveli National Science Foundation are working together to provide modest, but extremely important, funding to support the development of research on infectious diseases in Georgia. This endeavor needs understanding and continued support. Research, by its very nature, deals with the unknown, so results are slow in being realized, and the time it takes to obtain societal applications cannot be predicted and described with milestones incorporated into governmental plans. It is a challenge for scientist to put the value of research in terms to government decision makers that they appreciate and can utilize effectively in making their decisions.

The POE has always understood this conundrum and especially that the most important element of building an effective science program is educating and training young, creative scientists and to provide them with the opportunity to do their science in their home country. Considering the need for an educated workforce to fill jobs in the region that require a scientific expertise and to keep these young people focused on solving local problems of public and animal health, the challenge is huge. Many developing countries have not emphasized the need for education in the sciences and technical fields. Too often, they have seen this as an opportunity for their young people to leave the country to gain an education and find jobs elsewhere. International scientists need to help local governments build programs that are focused on solving local problems and contributing to the local economy and providing a reason for young scientists to stay involved at home.

Collaborative science is all about how to do the next good experiment, and it is also all about how to build trust and working relationships, with each other and with those in charge of seeing the program that is being developed has a future for the next generation of young scientists. Decision makers are beginning to recognize what enhancing science in the region can mean for economic development. Scientists need to do a better job in providing decision makers with facts as to how supporting a developing science program translates into lives saved and money well spent.

Another important lesson learned is that it is critical to step back on occasion to assess the appropriate course of action to develop science in a given region. The way western, namely, United States and European, scientists tackle problems may not always be the best for solving problems in the developing world. It is important to listen and understand that your approach may not always be the best for improving science and it needs to be adjusted to the situation and the time the work is ongoing. To enforce a singular point of view may be more harmful to the good relationships that are needed to do good science.

SECURITY

Because of the threat of bioterrorism, building an international science program dealing with infectious diseases with the goal of promoting threat reduction and global health security is, by its nature, a security challenge. Infectious agents have been and are likely to be used in the future by one people to do harm to another. In addition, concern for accidental exposure of a population to a pathogen, while scientific experiments are being conducted, cannot be ignored, even though modern laboratories and biosafety methods are coupled with aggressive training and have significantly reduced risk of accidental exposures.

An extremely difficult issue when trying to build scientific programs to study especially dangerous pathogens is whether acquiring fundamental knowledge of the mechanisms of action of pathogens represents a security risk. This is a question being grappled with by the research community around the world (7). While there is concern that fundamental knowledge can aid in nefarious development of a biological agent, the practicality is low. The biology of pathogenesis is so complex and modern methods of biological research have yet to match the capabilities of nature. So the benefits gained from knowing the workings of a pathogen and dealing with what nature is developing far outweigh potential negative consequences. The research is ongoing in the developed world, with participation of international students in some laboratories, and the results are publically available in the open literature. As a general statement, it can be said that research in the developing world needs to take advantage of publicly available knowledge and apply it to local challenges to improve the health of those people who need it the most. The portfolio of research that can be conducted in these developing laboratories needs to be allowed to match what is being done under approved regulations around the world and reported in the open literature. The scientific method and tools needed to acquire the knowledge are already at their disposal in the literature, on the internet and often when they are trained overseas, and to attempt to restrict participation in research based solely on country of origin would be misdirected.

SCIENCE EDUCATION

After the dissolution of the Soviet Union, education, particularly in the republics outside of Russia itself, suffered greatly. As Georgia, like other former Soviet Union republics began to pull itself out of the turmoil left during the post-Soviet struggle and as its educational institutes began to recover, science was not a priority in the education curriculae. The focus was more on those elements of an education that could immediately stabilize their economies. Education focused on where the jobs were and science jobs are not to be found in the post-Soviet republics. Those Georgians wishing to pursue careers in science were most likely to leave the country, to find science jobs elsewhere. The remaining scientists were those whose training was provided during Soviet times and this was mainly in Russia, along with the few younger students who were trained by these senior scientists. These scientists were trained to support centrally directed science programs with little opportunity for individual investigator driven, hypothesis-based research. It was difficult for a young scientist to become a leader of a research project. To build a science program in these countries without addressing the long term educational elements needed to train young scientists as creative thinkers is essentially a futile exercise. While a strong science education is important, it should be coupled with a place in the local economy for science based jobs. The public and animal health sector, together with support for research at the Universities to develop biotechnologies that help the local economy, is paramount for maintaining sustainable science.

Critical to the POE recommendations both with the SSA and the IP was building a new partnership with Georgian educational institutions. Traditionally, science in these countries during the Soviet times at research institutes was driven by directives from above and success was measured in the number of samples analyzed together with the analyses performed. Epidemiological analysis of the data was excellent, but for most scientists, discovery of fundamental new knowledge about the nature of the pathogens was not promoted. Samples were routinely taken to Russia for these more advanced studies. It was not that Soviet and post-Soviet scientists in the republics lacked the skills to perform hypothesis-driven research, they lacked the motivation. This approach to science has not gone away with the dissolution of the Soviet Union. Coupled with the explosive developments in life sciences around the rest of the world, this mentality has left post-Soviet scientist in the republics with a lack of confidence that they can compete for the funds necessary to carry out relevant research. Most support for research in these countries still comes from international sources because the local governments are focused on other elements of their economy. As the realization that long-term economic growth of the region depends on investment in education in science and technology, the struggle to catch up seems insurmountable, Added to this has been the increasingly competitive nature of generating research funding throughout the community of scientists. To counter this feeling

of being behind and having an impossible mission to catch up, the POE recommended a research program that would be uniquely theirs and would focus on ecological problems in their own neighborhood with factors that they uniquely could study and understand, but in turn is of global importance. The long list of newly emerging infectious diseases that come from local regions is evident that these problems are of global importance. Again the POE emphasized that for this program to be a success, there is a need to train young country scientists on how to generate creative new ideas on how to solve important problems. Such training would involve working closely with highly successful scientists from around the world not for just a short period of time, but long enough to learn what it means to come up with a hypothesis and design an approach to test it. There needs to be provided seed money to support the effort of these newly trained scientists to succeed or fail with their own hypotheses. The recommendation is a two-part program of international training along with support to place successful international scientists at the CPHR on sabbatical and work at the Lugar Center along with young Georgian scientists. These types of collaborations bring long-term relationships to be applied to any new infectious disease outbreak that might occur.

Improving science education in the post-Soviet republics remains a long-term goal and can be achieved with the help of international scientists. However, it cannot move forward without a commitment from the local government. The POE supports the goals of international agencies working to help the Georgian government improve education in the sciences by working with organizations like the Millenium Challenge Corporation (8) and the European Union. The POE University training program has been working with the Fogarty International Center of the National Institutes of Health, as well as European Union organizations, to provide support for Georgian students to work in international laboratories and also maintain affiliation with their home institution. This training includes at least a year spent working in the international laboratory. A major requirement is that the student develops and submits a research proposal for a project to be conducted at home in support of the global health security.

COLLABORATIVE SCIENCE

Collaborative science in the developing world is not the same as that in Europe or the West. In a setting where science is well developed, collaborative science is working together of research teams with different, but supportive capabilities to address and solve a question that neither group alone can accomplish. In the developing world, collaborative science with participation of international experts must take on a much more inclusive role. The goal is to promote development of modern science for individuals in the host country, not for the financial gain or career development of the international scientist. This is a particularly sensitive issue for both host governments and for host scientists. And if the situation is not absolutely clear, it leads to loss of trust on the part of those whom the program intends to aid. International collaborating scientists must be sure to focus on benefit to the participating scientists in the developing region to ensure a positive impact on science in these regions. This includes appropriate authorship on manuscripts, export of research materials back to western or European laboratories, or hiring away talented young students needed to sustain the program of the developing countries instead of developing mutual partnerships between laboratories. It is particularly difficult for international scientists to remain behind the scenes when on contract by international agencies and their contract depends on showing evidence of their work on the given project. However, this fact is not lost to host country participants and it makes them question the motivation of the international effort. The long-term benefit to science of genuine and full partnership for the sake of science is much more important than any short-term benefit to an individual or corporation.

MISSION RESTRICTIONS

The mission space of most funding agencies does not allow for the support of all aspects of a program needed in developing countries to make their mission a success. In regions like the former Soviet Union republics where support of science has been lacking for so many years, building a successful program that can achieve health security and compete for funding in the modern world requires a complete redevelopment of the education system and patient financial support, while a critical mass of scientists can come together to generate new understanding of scientific research relevant to the region. In the area of infectious diseases, this is particularly difficult, not because of the science itself being complex which of course it is, but because of politics. It is important that the local government is a stakeholder in the science program and is committed to see it succeed.

As discussed earlier, working on issues that impact global health security in developing countries presents funding agencies with some difficult problems. While the goal of any program dealing with health security and infectious disease may be clear to the scientists involved, assembling components to make a sustainable meaningful effort is a challenge. It takes more than building laboratories and supplying modern equipment and training in the operations of the systems. It takes building an understanding and commitment to address what is needed. Because of the dynamic nature of infectious agents, whether they originate in nature or from nefarious activity, it requires creative research to be able to respond in a timely manner to contain spread of infection, save lives, and prevent economic disaster. Research at the local level is of prime importance. That research must be supported by funding agencies led by a host government that understands the need for patience in developing a capacity for research on infectious disease.

While the overall goal is to support threat reduction and global health security through science, programs usually operate within their own guidelines that may limit the possibility of achieving the larger goals. Focusing on one part of the problem leaves institutions having to piece together support to maintain facilities, trained scientists, and program goals. Nature does not work from a list of select agents. In the modern world, potential bioterrorists are not restricted to a "Cold War" list of agents to inflict damage to their enemy. Threat reduction in the developing world and globally comes not from restricting scientists to a narrow definition

of understanding disease and devising the means for saving lives, but it comes from attacking those problems impacting the health of people in their own neighborhood with creative solution and working to prevent similar outbreaks elsewhere and in the future. Understanding the mechanisms of action of any one pathogen helps in the understanding of all such agents. Next generation nucleic acid sequencing technologies have shown how complex the microbial community really is, but there is yet to be unraveled the overall system of how these agents have come to be, how they operate, and where they are going. The list of ecological factors that shape these outbreaks represents a hugely complex system and ever changing. Understanding these processes needs to start locally and expand globally and will allow us to deal with immediate outbreaks and predict future events.

The ISAC and POE have worked to support development of a relevant and contemporary science program at the CPHR in the Republic of Georgia for ~3 years. The work is far from completed. The POE effort has succeeded in stimulating new scientific ideas on the part of Georgian scientists and has introduced them to new potential collaborators. The work accomplished to date has also challenged the Georgian scientists to consider those issues faced by their country in a new dimension and they have learned how to develop research proposals with themselves as principal investigators. They have joined the community of scientists who are finding universally, that funding for good ideas is hard to find, and the search is frustrating. Workshops on important topics have provided focal points for scientific development in the region and have brought together international experts with excellent discussion and new ideas for projects, none of which have yet been funded but the potential (and hope) remains.

The experience of the POE has proven that there is no shortage of excellent scientists and agencies dedicated to building scientific capacity in developing countries focused on the improvement of public and animal health. All are committed to making a difference, but the agencies operate within the confines of their own missions, and effective integration of these well-intentioned efforts is very much needed.

A key truism is that one cannot predict where the next good idea will come from. And that is certainly is the case in addressing the threat of infectious disease. It is the human component of the equation that matters most and makes the reward of building science programs in the developing world a certainty. It is an investment in the future.

Over the 3 years, the POE has worked diligently to promote research and research capacity for gaining understanding of pathogens in this corner of the world, nature has conducted trillions upon trillions of "experiments" to develop new versions of agents and to challenge life on our planet, all while the world has become a less stable place.

ETHICS STATEMENT

Our study was carried out in accordance with the rules set forth in the Federal Advisory Committee Act of 1972. All members of the Panel of Experts were consultants to the Defense Threat Reduction Agency.

ACKNOWLEDGMENTS

The authors would like to especially thank all the Georgian scientists who graciously shared their ideas and time with us and our workshop participants. And without the enthusiastic participation of our workshop participants much would have been lost from all the discussions. We would also like to thank Allison Bigbee, Jason Farlow, and Justin Bartholomew for critically reading the early drafts of this manuscript and for many useful discussions.

FUNDING

The activities of the POE were supported contract HDTRA1-13-C-0076 subcontract 4949-JCB-DTRA-076 through The Pennsylvania State University.

REFERENCES

1. Lederberg J. (2003). Available from: http://www.digitalnpq.org/global_services/nobel%20laureates/04-21-03.html
2. National Academy of Sciences/Institute of Medicine/National Research Council. *Controlling Dangerous Pathogens: A Blueprint for U.S. – Russian Cooperation* (1997). Available from: http://www.nap.edu/read/9471/chapter/1
3. Lerner H, Berg C. The concept of health in One Health and some practical implications for research and education: what is One Health? *Infect Ecol Epidemiol* (2015) **5**. doi:10.3402/iee.v5.25300
4. Konstantinidis KT, Ramette A, Tiedje JM. The bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* (2006) **361**:1929–40. doi:10.1098/rstb.2006.1920
5. Rahaman MH, Islam T, Colwell R, Alam M. Molecular tools in understanding the evolution of *Vibrio* cholera. *Front Microbiol* (2015) **6**:1040. doi:10.3389/fmicb.2015.01040
6. Fogerty International Center Newsletter. (2013). Available from: http://www.fic.nih.gov/news/globalhealthmatters/march-april-2013/pages/cugh-university-global-health-programs.aspx
7. Evans NG. Dual-use decision making: rational and positional issues. *Monash Bioeth Rev* (2014) **32**:268–83. doi:10.1007/s40592-015-0026-y
8. Millenium Challenge Corporation Website. (2015). Available from: https://www.mcc.gov/pages/countries/program/georgia-compact-ii

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

PERSPECTIVE
published: 14 July 2015
doi: 10.3389/fmicb.2015.00681

Cross-cultural science: ten lessons

*Joanne M. Horn**

Global Bioengagement, MRIGlobal, Frederick, MD, USA

Concerns of infectious disease outbreaks have recently reached the forefront of global security issues and resulted in new engagements among foreign science advisors, host country scientists, and officials. There are lessons to be learned from the numerous organizations working in global regions of endemic disease who are building capacity to survey pathogens and prevent and contain epidemics. Working with foreign scientists, health professionals, and administrators can be challenging; building partnerships based on respect and mutual trust is key to achieve effective change. Engendering ownership, working toward mutual success, paying close attention to cultural norms and the local regulatory climate, close collaboration with other stakeholders, and imaginative problem solving all contribute to mission success.

Keywords: bioengagement, partnering, capacity building, biosurveillance, global health security, disease outbreak prevention

OPEN ACCESS

Edited by:
Jeanne M. Fair, Defense Threat Reduction Agency, USA

Reviewed by:
Paras Jain, Albert Einstein College of Medicine of Yeshiva College, USA
Martha M. Stokes, Cooperative Biological Engagement Program, USA

***Correspondence:**
Joanne M. Horn, Global Bioengagement, MRIGlobal, 110 Thomas Johnson Drive, Suite 170, Frederick, MD 21702, USA
hornj19@gmail.com

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *28 January 2015*
Accepted: *22 June 2015*
Published: *14 July 2015*

Citation:
Horn JM (2015) Cross-cultural science: ten lessons. Front. Microbiol. 6:681. doi: 10.3389/fmicb.2015.00681

Introduction and Background

In recent years, many factors have converged that have collectively increased the threats of pandemic infectious disease. The globalization of trade and travel, expanding use of wildlife resources and habitats by humans, cultural practices bringing livestock and people into close contact, episodes of war and terrorism, and climatic change have focused our collective awareness of the continuing need for engagement to strengthen global health security. A number of national and international programs are operating throughout the world with mandates to sustainably improve biosafety practices, disease surveillance capacity, outbreak response and reporting, and supporting and sharing research activities. These include such organizations as Global Health Partnership; the European Union Chemical, Biological, Radiological, and Nuclear Centers of Excellence Initiative; the United Nations-sponsored groups, World Health Organization, the World Organization for Animal Health, and Food and Agriculture Organization; and U.S. Government agencies spanning the Departments of Defense, State, Health, and the U.S. Agency for International Development. Other organizations such as the President's Emergency Plan for AIDS Relief (PEPFAR) and the Global Fund to Fight AIDS, Tuberculosis, and Malaria have been instrumental in targeting specific infectious disease that affect a broad swath of humanity in the developing world. The Global Health Initiative has sought to strengthen public health systems worldwide. In pursuit of these goals, global health professionals have fanned out across the globe, establishing relationships with local infectious disease scientists, clinicians, research institutes, public health laboratories, hospitals, and government officials in focus countries and regions.

Many of these efforts have been guided by the evolution of the goals of global health policy since the end of the Cold War, from those seeking to advance political and economic advantage, to seeking security and stability (Barnes and Brown, 2011). Driven by deliberative-based theories of inclusiveness for driving collective decisions (Brown, 2010), and practical awareness of effective methods to achieve development goals, the principle of establishing true partnerships with aid recipients was actively promoted in the mid-1990s (Barnes and Brown, 2011). The following decade brought the concepts of pursuing target country ownership of health programs, along with a more

or less defined path toward capacity building: to assess, plan, monitor, and evaluate (Goldberg and Bryant, 2012). Along the way, many challenges and requirements have been documented to make these principles work, including jointly defining goals and metrics, the transference of operations to local control mechanisms, and the need to integrate the efforts of all aspects of public health infrastructure with disease detection (Garrett, 2007).

MRIGlobal, as a (not-for-profit/independent research) contractor, has been providing support for the Defense Threat Reduction Agency's Cooperative Bioengagement Program for the last 10 years, and also helped support global health security missions in the Middle East, South Asia, and Africa over the last 20 years. Executing these science-based programs has put us into direct continuing contact with local collaborators in infectious disease science, and consistently reinforced the absolute requirement to establish collegial relationships with our foreign partners to successfully execute programmatic objectives. Along the way, we have learned many lessons—some hard won—in communication and understanding across the cultural divide, and gained important insights from the many individuals engaged in these programs. We share here some of what we and others have discovered as an aid in the ongoing development of effective mechanisms for the near term, and to ultimately reduce the infectious disease burden globally.

Our experience is certainly not unique. Many other accomplished professionals have applied serious analysis and relayed formulations to successfully improve the capacity for detecting, responding and preventing infectious disease (or other risks, such as nuclear threats) throughout the developing world. These particular guidelines were chosen because they are fundamental, and so generally apply to human relationships across the board, in business, policy, or technology; but they seem especially essential for successfully changing views and behaviors across vastly different cultures. The reward of success in these programs, as Harold Varmus states, "is a connection made to the culture of science, in which decisions are based on evidence. . .and interactions among people of varied backgrounds are grounded in common goals" to improve global health security (Varmus, 2014). Investing in these relationships, in short, can help improve the health and well-being of countless lives, and bolster our collective security from disease risks.

Guides to Constructive Partnering

(1) **Mutual respect is foundational.** While corporate and academic environments in the U.S. may lend themselves to productive adversarial or competitive approaches as key to success, these are counterproductive in establishing working relationships in most foreign cultures. Approaching potential partners with an appreciation and open respect of their knowledge and skills builds mutual understanding and trust. As has been noted, global health professionals need to value individual and organizational representative capacity, as well as technical competence (Kevany, 2014), and function as capable health diplomats to be successful (Novotny and Adams, 2007). A partners' willingness to cooperate, listen, and follow through with meaningful action is ultimately rooted in shared trust. No matter how profound the individual differences may seem, a personal connection is valuable when seeking mutual areas of interest and investment, to allow all parties to reach a position of shared purpose.

One indicator of achieving trust is a continued partnership, which can offer reward. A scientist who engaged a national Laboratory in West Africa and helped solve their cold-chain supply issues by facilitating installation of a nitrogen generator, was later called back to respond to the Ebola crisis and provided an "incredible amount of data" that wasn't accessible previously (Rozo, 2014). Trusting partnerships can provide low-key cooperative relationships even while government-to-government negotiations are in flux, and offer reality checks on what truly constitutes a security risk (Franz and Lehman, 2009).

(2) **Ownership is essential.** "Country Ownership" is a capacity building strategy that shifts the leadership and responsibility for enacting systematic improvements onto the partner country and includes local involvement (Goldberg and Bryant, 2012). The most effective way to affect change is for a partner to adopt your goals and objectives as their own. They will ideally realize that outcomes will enable advances for individual and societal benefits—whether social, personal, political, economic, or strategic. Partners should take possession of the cause and advance it as their own. This "personalization of objectives" is a critical step toward sustainable programs. Without ownership, the associated programs and objectives will be abandoned once the foreign sponsor ceases involvement. Partner investment and ownership is essential to embed programs into national science and governance, and true partnerships are requisite to conduct systematic assessments, analyze challenges, define goals, prioritize action, and implement activities (Goldberg and Bryant, 2012).

In June, 2012 a group of U.S. science and engineering students and an experienced group of researchers from Yemen came together in Jordan under the auspices of the Federation of American Scientists' International Science Partnership. As they worked through issues to address Yemen's energy and water dilemmas it became clear that the Yemeni scientists were not only interested I furthering humanitarian concerns, but also "exploring new ideas, learning from peers, and gaining experience to further their own careers" (Jansson and Ferguson, 2012). While this is not surprising, it does demonstrate that ownership can be achieved from a number of directions.

(3) **Remain perceptive.** While the term "cultural sensitivity" has become a cliché, it is vital to appreciate why people speak or act as they do. Listen actively and cultivate an awareness of nonverbal cues such as body language; and consult with sympathetic local nationals to get an idea of whether cultural customs may be playing a part in your interactions (Katz et al., 2014). By a common example, it is considered polite to exchange pleasantries and inter-personal banter prior to starting a meeting. Despite our Western urges

to get on with the agenda and limit our introductions to 30-s elevator speeches, this approach is widely considered rude by others. Regarding hierarchical systems, an individual's professional background determines the credence given to all later statements in a meeting. Also, due respect must be paid to senior members of any delegation. Partners can be protégés of their respective patrons throughout their careers—they may have little individual power to make decisions without the full agreements of their boss. Overall, developing a knowledge of foreign cultures, values, and norms is key to success (Katz et al., 2014) and adaptation to local needs and customs is often essential (Kevany et al., 2012).

(4) **All the players matter.** Efforts to build and sustain programs depend not only on the immediate relationships you build with local individuals but also with governmental representatives, non-government organizations, international organizations, other agencies, and contractors (Atun and Kazatchkine, 2009). There is often much to learn from fellow stakeholders, and cooperation helps avoid wasteful duplication of effort while working toward mutual goals; multilateral coordination is very helpful (Katz et al., 2014). Other organizations—especially well-established ones—can frequently help navigate the terrain if mutual wins are sought. Despite these efforts, the interests of individuals, organizations and governments may fail to align and the net benefits may result in their unequal distribution creating both winners and losers (Smith, 2014). In some situations, local institutions may try to disrupt or prevent communication between different stakeholders. Only frequent, independent, often confidential, exchanges between stakeholders can prevent double-dipping by recipients and allow stakeholders to best leverage collective resources and find synergies.

(5) **Ethical conduct is key.** Do not promise what you cannot deliver; if you need to consult the sponsor, upper management, or other stakeholders, then disclose that fact; be transparent. There are many instances where there is misunderstanding of the uncertainty of the flow of knowledge globally (Anderson, 2002), which might be avoided by a clear explanation of how, why, and where knowledge is disseminated. Scrupulous attention to fairness pays; often, local institutions are competing for scarce resources. Limit your discussion to the institution you are dealing directly with to avoid political infighting. On the institutional level imparting the "3 Cs of Biosecurity: Codes of Ethics, Codes of Conduct, and Codes of Practice" (Nasim et al., 2013) starts with the relationship between individuals; be an example of what you hope to create.

(6) **Work-arounds to rejection.** Confronting a wall of refusal, denial, deception, or other misdeeds is frustrating and can be threatening to mission success. While the urge may be to directly confront the individuals delivering the message, active listening to understand the underlying issues may be more productive (Katz et al., 2014). There may be alternative explanations or motivations that lend themselves to resolution. For example, management or policy forces your partner to react, local law prevents action, or traditional corruption or patronage pathways are obstructive. Often, approaching the immediate partner face-to-face and asking what might be acceptable to their management, along with offering alternatives, is a successful path to conflict resolution. For example, we were once confronted with refusal to export microbial strains from a country we engaged with, preventing any progress on an entire joint research program. We worked out alternatives—raw sample transfer, DNA transfer, data transfer—and asked our local project collaborators what they thought their ministries would accept. Our sponsors took that information to the ministries, and we were able to negotiate a compromise. In summary: Stay flexible and develop alternate plans for reaching your goals.

As a corollary, be careful if you are considering citing someone's support for a concept directly to their management. In other words, do not argue to upper levels of management that they should buy into an idea because their underlings agree. This approach may fly I the face of hierarchal decision-making and backfire, as well as put allies at risk.

In a time when pathogen discovery and disease disclosure can have serious political, diplomatic, economic, or military implications, extreme caution needs to be exercised in approaching the right authorities with the correct information, and offer constructive, accurate, and actionable solutions. The emergence of influenza strain H5N1 in Indonesia, for example, caused the sequestration of critical viral samples ("viral sovereignty," among other downstream events, Elbe, 2010; Smith, 2014); such reactions can potentially have disastrous consequences.

At the end of the day, health diplomats whether clinical, scientific or policy-makers, need an understanding of the structures, programs, approaches, and pitfalls of their relationships (Novotny and Adams, 2007). As one illustration, the International Science and Technology Center (ISTC), an multi-country consortium that seeks to counter proliferation, has had their projects curtailed in Russia due to political mistrust. This situation may be compounded by the demise of influence of scientists and their influence on policy (Bergstrom, 2011).

(7) **Don't rely on individuals.** Political and management structures can rapidly change, and while you take time to build and cultivate good interpersonal relationships, trust is not necessarily a transferable commodity (Smith, 2014). Expect to educate new players as they come onboard; this may also translate to educating their entire staff. Patronage systems often involve wholesale change: When one chief is dismissed, all the other subordinates can fall, too. It has also been pointed out that whatever legitimacy is forged among scientists must be transferred to policy setters to have a larger effect, and that transmission requires connectivity; trust can be difficult to transfer (Smith, 2014). Harold Varmus has in fact proposed that while admirable efforts to build capacity in Uganda and Mali, these have been overtaken at times by larger political forces (Varmus, 2014).

(8) **Know the regulatory context.** Understand limitations of local and national statute; you will need to work within these to keep the program from running aground and your partners cooperative. The Center for Science, Technology, and Security Policy (AAAS) implemented a cooperative research grant program in the broader Middle East and North Africa in 2010, and specifically pointed out that areas of concern may range from ethical research practices, to export control, contract law, and transport regulations (Coat et al., 2013). These issues become magnified when regional projects involving more than one country are encountered. The formulation of new laws may be possible if the right people and institutions can be involved; or if it is possible to incorporate important revisions of current laws by merging international norms with current local law.

(9) **Pay Attention to metrics.** Programmatic metrics and evaluations of effectiveness are important when gaging success —it is equally critical to impart these skills and approaches to target partners. Selecting key indicators of success and unbiased self-evaluation will help to continually improve programs, even after the sponsor is gone. As with other aspects of engagement, flexibility is key. If the engaged partners are not willing to share a key piece of information out of security or other concerns, try to get agreement on an alternate parameter that is acceptable. It has been reasonably argued that a body of evidence –based metrics should be built around what works to build disease surveillance and outbreak response (Goldberg and Bryant, 2012). Identify indicators that measure change over time (Honadle, 1981) and are truly representative and reflect the goals of the program as identified in the initial assessment phase and promote targeting areas that require further investment. Indicators can vary and include process, output and impact indicators (Goldberg and Bryant, 2012). While it can be difficult to identify appropriate indicators for "soft" program results, such as improving prioritization of risk, these are critical to assess (Franz and Lehman, 2009).

(10) **Outliers can be central.** Institutions or individuals who are not primary programmatic or contractual targets may end up being fundamental to achieving sustainability. If you approach these players, there can be big payoffs. Never turn away a potential participant without carefully considering what they can bring to bear. If you approach them independently, they may well be honored—and there may be benefits to them and their institutions. Such is often the case, for example, with universities educating the pipeline of new leaders. Makerere University's Ugandan Cancer Institute survived through the Amin dictatorship largely due to the heroic efforts of Charles Olweny, who was mentored early in his career by colleagues at the U.S. National Cancer Institute and Sweden's Karolinska Institute (Varmus, 2014). Strive for a broad but effective reach within the limitations of schedule and budget, and with sponsor approval.

Engaging with foreign cultures to generate cultural scientific change and promote genuine improvements in human and animal health can be both frustrating and rewarding. Continuous recycling back to the same issues, dealing with a revolving door of officials, stonewalling, and sometimes plain abject rejection are all challenging. However, accept that change will be incremental and that good ideas often take years to have an impact, even in our own systems. Some estimate that it will take at least a full generation or more to substantially improve public health in some of the developing world (Garrett, 2007). Maintain patience; keep educating as new players enter the scene. Stay positive; soured relationships rarely turn around once mistrust is established. If you have the opportunity to work long-term on a program, there is satisfaction in the long view back, where friendships have been forged, colleagues gained, and the path toward change is being realized.

References

Anderson, W. (2002). Introduction: postcolonical technoscience. *Soc. Stud. Sci.* 32, 643–658. doi: 10.1177/030631270203200502

Atun, R., and Kazatchkine, M. (2009). Promoting country ownership and stewardship of health programs: the global fund experience. *J. Acquir. Immune. Defic. Syndr.* 52(Suppl. 1), S67–S68. doi: 10.1097/qai.0b013e3181bbcd58

Barnes, A., and Brown, G. W. (2011). The idea of partnership within the Millennium Development Goals: context, instrumentality and the normative demands of partnership. *Third World Quar.* 32, 165–180. doi: 10.1080/01436597.2011.543821

Bergstrom, L. (2011). "*The Dying Voice of cooperative Nonproliferation's Greatest Advocates.* ed. The Nonoproliferation Review, Copyright 2014, James Martin Center for Nonproliferation Studies. Available at: http://wmdjunction.com/111116_dying_voice.htm

Brown, G. W. (2010). Safeguarding deliberative global governance: the case study of the Global Fund to Fight AIDS, Tuberculosis and Malaria. *Rev. Intl. Stud.* 36, 1–20. doi: 10.1017/S0260210510000136

Coat, G., McArdle, J., Marburger, L., and Berger, K. (2013). *Report and Proceedings from the 2010–2012 AAAS Middle East and North Africa Meeting Series, 'International Engagement: Bioscience for a Safe and Secure Society," Scientific Engagement in the Broader Middle East and North Africa: Lessons Learned in promoting a Safe and Secure Research Environment*. Available at: http://www.aaas.org/report/scientific-engagement-broader-middle-east-and-north-africa-lessons-promoting-safe-and-secure

Elbe, S. (2010). The downside risks of securitizing infectious disease. *Health Policy Plan.* 25, 476–485. doi: 10.1093/heapol/czq050

Franz, D. R., and Lehman, R. II. (2009). *Committee on Strengthening and Expanding the Department of Defense Cooperative Threat Reduction Program, National Academy of Sciences, Global Security Engagement: A New Model for Cooperative Threat Reduction*. Washington, DC: The National Academies Press.

Garrett, L. (2007). The challenge of global health. *Foreign Affairs*. Available at: http://www.foreignaffairs.com/print/62268

Goldberg, J., and Bryant, M. (2012). Country ownership and capacity building: the next buzzwords in health systems strengthening or a truly new approach to development? *BMC Public Health* 12:531. doi: 10.1186/1471-2458-12-531

Honadle, B. W. (1981). A capacity building framework: a search for concept and purpose. *Public Adm. Rev.* 41, 575–580. doi: 10.2307/976270

Jansson, M., and Ferguson, C. D. (2012). *Building a New Foundation with Yemen. Science and Diplomacy, AAAS*. Available at: http://www.sciencediplomacy.org/perspective/2012/building-new-foundation-yemen

Katz, R., Blazes, D., Bae, J., Putambekar, N., Perdue, C. L., and Fischer, J. (2014). Global health diplomacy training for military medical researchers. *Mil. Med.* 179, 364–369. doi: 10.7205/MILMED-D-13-00389

Kevany, S. (2014). Global health diplomacy: 'Smart Power', and the new world order. *Global Public Health* 9, 787–807. doi: 10.1080/17441692.2014.921219

Kevany, S., Khumalo-Sakutukwa, G., Murima, O., Chingono, A., Modiba, P., Gray, G., et al. (2012). Health diplomacy and the adaptation of global health

interventions to local needs in sub-Saharan Africa and Thailand: evaluating findings from Project Accept (HPTN 043). *BMC Public Health* 12:459. doi: 10.1186/1471-2458-12-459

Nasim, A., Al-Hmoud, N. D., AlMomin, S., Rashid, N., Temsamani, K. R., Berger, K., et al. (2013). *Paths to Biosafety and Biosecurity Sustainability, Science and Diplomacy, AAAS*. Available at: http://www.sciencediplomacy.org/print/136

Novotny, T., and Adams, V. (2007). Global Health Diplomacy—a call for a new field of teaching and research. *San Franc. Med.* 80, 22–23.

Rozo, M. (2014). *Placing Global Biosecurity Engagement Programs Under the Umbrella of Global Health Security. Federation of American Scientists Issue Brief*. Available at: http://fas.org/wp-content/uploads/2014/05/bio-engagement-final-may-2014.pdf

Smith, F. L. III. (2014). Advancing science diplomacy: Indonesia and the US Naval Medical Research Unit Soc. *Stud. Sci.* 44, 825–847. doi: 10.1177/0306312714535864

Varmus, H. (2014). *Medical Research Centers in Mali and Uganda. Science and Diplomacy, AAAS*. Available at: http://www.sciencediplomacy.org/article/2014/medical-research-centers-in-mali-and-uganda

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

MINI REVIEW
published: 01 February 2016
doi: 10.3389/fpubh.2016.00004

Biosurveillance in Central Asia: Successes and Challenges of Tick-Borne Disease Research in Kazakhstan and Kyrgyzstan

John Hay[1], Kenneth B. Yeh[2]*, Debanjana Dasgupta[2], Zhanna Shapieva[3], Gulnara Omasheva[3], Pavel Deryabin[4], Talgat Nurmakhanov[4], Timur Ayazbayev[5], Alexei Andryushchenko[5], Asankadyr Zhunushov[6], Roger Hewson[7], Christina M. Farris[8] and Allen L. Richards[8]*

[1] State University of New York, Buffalo, NY, USA, [2] MRIGlobal, Rockville, MD, USA, [3] Scientific Practical Center for Epidemiological Expertise and Monitoring, Almaty, Kazakhstan, [4] Kazakh Scientific Center for Quarantine and Zoonotic Diseases, Almaty, Kazakhstan, [5] Uralsk Anti-Plague Station, Uralsk, Kazakhstan, [6] Biotechnology Institute, Bishkek, Kyrgyzstan, [7] Public Health England, Salisbury, UK, [8] Naval Medical Research Center, Silver Spring, MD, USA

OPEN ACCESS

Edited by:
Nathan Wolfe,
Metabiota, USA

Reviewed by:
Li Xu,
Cornell University, USA
Brad S. Schneider,
Metabiota, USA

***Correspondence:**
John Hay
jhay150@gmail.com;
Kenneth B. Yeh
kyeh@mriglobal.org

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: 29 May 2015
Accepted: 11 January 2016
Published: 01 February 2016

Citation:
Hay J, Yeh KB, Dasgupta D, Shapieva Z, Omasheva G, Deryabin P, Nurmakhanov T, Ayazbayev T, Andryushchenko A, Zhunushov A, Hewson R, Farris CM and Richards AL (2016) Biosurveillance in Central Asia: Successes and Challenges of Tick-Borne Disease Research in Kazakhstan and Kyrgyzstan. Front. Public Health 4:4. doi: 10.3389/fpubh.2016.00004

Central Asia is a vast geographic region that includes five former Soviet Union republics: Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan. The region has a unique infectious disease burden, and a history that includes Silk Road trade routes and networks that were part of the anti-plague and biowarfare programs in the former Soviet Union. Post-Soviet Union biosurveillance research in this unique area of the world has met with several challenges, including lack of funding and resources to independently conduct hypothesis driven, peer-review quality research. Strides have been made, however, to increase scientific engagement and capability. Kazakhstan and Kyrgyzstan are examples of countries where biosurveillance research has been successfully conducted, particularly with respect to especially dangerous pathogens. In this review, we describe in detail the successes, challenges, and opportunities of conducting biosurveillance in Central Asia as exemplified by our recent research activities on ticks and tick-borne diseases in Kazakhstan and Kyrgyzstan.

Keywords: tick-borne diseases, TBE virus, *Rickettsia*, *Coxiella*, Kazakhstan, Kyrgyzstan, biosurveillance

BACKGROUND

Biosurveillance research and environmental monitoring have been conducted in Central Asia since the Russian anti-plague (AP) network in the 1890s set up by Czar Nicholas II (1). This network was originally organized geographically, to conduct surveillance of local diseases, such as plague, and to prevent introduction of diseases with no natural foci, such as cholera (2). During the Soviet era, the AP network evolved into a highly structured organization that included over 100 facilities: observational, field, regional stations, and institutes, across the 11 republics that were administered by the Union of Soviet Socialist Republics' Ministry of Health (3). After the Soviet Union collapsed, each country maintained its own AP system that varies by country (4). All have experienced challenges of limited funding and lack of training for their specialists, while competition for limited funds

has decreased collaboration as well as development of programs fostering peer-reviewed quality research and related funding.

Two countries that exemplify the current status of biosurveillance in Central Asia are Kazakhstan and Kyrgyzstan. Kazakhstan (2.7 million square kilometer; 18 million people) has national wealth in the forms of oil, natural gas, and mineral resources[1] but still relies heavily on imported expertise and technology to further develop their resources (5). Kyrgyzstan (0.2 million square kilometer; 5.7 million people) has no oil or natural gas but has mineral resources (see text footnote 1), and also relies on imported expertise and technology (6) (**Figure 1**). In addition to commercial ventures with foreign petroleum companies, aid to both countries has come from the United States Department of Defense (US DoD) cooperative threat reduction (CTR) program, through the 1991 Soviet Nuclear Threat Reduction Initiative. The governments of both countries are cooperating on biological threat reduction efforts, with an emphasis on disease surveillance, biosafety, and biosecurity. As with other supporting nations, aid from the United States (US) first requires a formal government-to-government (umbrella) agreement, which establishes diplomatic intent and cooperation. Next, an implementing agreement is established with a specific US government agency. In Kazakhstan, an umbrella agreement was signed in December 1993 and the implementing agreement was signed in 1995. The cooperation includes a broad range of nuclear security and non-proliferation topics. Kyrgyzstan has also signed bilateral investment and trade agreements with the US. In Kyrgyzstan, multiple foreign agencies have been involved in projects utilizing the country's AP system and run through the Republic Center of Quarantine and Especially Dangerous Infections (RCQEDI) (4). For example, International Science and Technology Center (ISTC), Civilian Research and Development Foundation (CRDF) Global, United States Department of State (US DoS), and the

[1] https://www.cia.gov/library/publications/the-world-factbook/geos/kg.html

FIGURE 1 | Commonwealth of independent states – Central Asian States. Reproduced with permission from (6).

United Kingdom Ministry of Defense (UK MoD) have all set up collaborative biosurveillance projects with local scientists.

Historically, tick-borne diseases have been important public health issue in Central Asia, and biosurveillance has involved regular collection and archiving of tick samples. Collection locality information associated with these samples has already contributed to vector biogeography databases, such as VectorMap.[2] Analyses of these frozen tick samples (e.g., molecular testing for evidence of infection by pathogens) offer numerous opportunities for additional research and collaboration, some examples of which are described below. In a recent paper, Han et al. (7) predicted the existence of a large reservoir of undiscovered zoonotic infections in this part of the world.

As examples of the accomplishments and challenges of biosurveillance research conducted in Central Asia we present below, as case studies, our work on ticks and tick-borne diseases conducted in Kazakhstan and Kyrgyzstan over the past decade. This work includes studies of tick-borne encephalitis (TBE) in both counties and of rickettsial diseases, Q fever, Crimean Congo hemorrhagic fever (CCHF), and hemorrhagic fever with renal syndrome (*Hantaviruses*) in Kazakhstan. For reasons of space limitation, we will not discuss the CCHF (8) or *Hantavirus* work in Kazakhstan (9) here.

TICK-BORNE ENCEPHALITIS

Tick-borne encephalitis is caused by a flavivirus (TBEV); there are three types: European, Siberian, and Far-Eastern. Of these, the Far-Eastern strain causes the most serious disease. Infection is spread to humans through tick bites and through ingestion of raw milk and milk products (10). Wild and domestic animals may be hosts for the virus (10). TBE is endemic in a wide range of European and Asian countries, and effective vaccines are available in many countries (11). Anecdotal information and papers in the local literature suggested that the virus and the disease may be more widespread than was understood, in particular, that the virus may range in more southerly locations in Asia (11, 12).

Based on this, we initiated a series of studies in Kyrgyzstan. We found TBEV in the taiga tick (*Ixodes persulcatus*) and in rodents including the Himalayan field mouse (*Apodemus pallipes*), a previously unknown TBEV host (13). Sequencing studies showed this Kyrgyz virus to be a close relative of a Siberian strain from Novosibirsk (13). We also identified the virus in a sample from a fatal case of TBEV involving a hiker who had visited an area where we found the virus in ticks (13). Thus, TBEV and TBE appear to occur much further south (42.6°N) and at much higher altitudes (~2,100 m) than previously believed (13).

Following this, we extended our TBE/TBEV research into Kazakhstan to identify and characterize TBEV there. This work, part of collaboration with Kazakhstani colleagues from three government agencies, has yielded several interesting findings over the past decade. Using modern molecular methods and an epidemiologically based approach, we tested human and animal samples from selected areas where TBE is known or suspected to occur and found strong evidence that TBEV does circulate in Kazakhstan Scientific colleagues from the Kazakh Scientific Center of Quarantine and Zoonotic Diseases (KSCQZD), Scientific Practical Center for Sanitary Epidemiological Expertise and Monitoring (SPCSEEM), and Uralsk Anti-Plague Station (UAPS) collaborated to provide samples and associated collection data, and assist with diagnostics. UAPS staff focused its work on tick samples from West Kazakhstan and Aktobe oblasts; KSCQZD staff collected ticks from Almaty, Kostanay, Kyzylorda, Mangystau, South Kazakhstan, and Zhambyl oblasts; and SPCSEEM staff obtained ticks from Atyrau, East Kazakhstan, Karaganda, North Kazakhstan, and Pavlodar oblasts. Collectively, tick samples included over 40,000 specimens collected from 13 of Kazakhstan's 14 oblasts (i.e., provinces). Samples from captured rodents and stored human sera were shared by staff of UAPS and SPCSEEM, respectively. Our US-based staff provided technical guidance to Kazakh to facilitate confirmation of morphological tick identification by molecular methods [e.g., polymerase chain reaction (PCR) and pooling and testing of ticks for infection by TBEV, CCHFV, and *Rickettsia* spp. by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time PCR (qPCR)].

As we hypothesized, TBEV is more widespread in Kazakhstan than was previously believed. Areas in the southern and eastern regions harbor infected ticks (14). Unfortunately, we were unable to get material from the western region, where we have strong suspicions that the virus also circulates. In the southern and eastern regions, data from 2005 to 2014 show, on average, 35 cases or 0.22 cases per 100,000 per year (15). This burden of disease apparently occurs in spite of the fact that up to 50,000 doses of vaccine are administered in endemic areas each year. Additionally, we found evidence of TBEV infection in ticks in the genera of *Dermacentor*, *Hyalomma*, and *Haemaphysalis* (14, 16, 17). The collective range of these ticks is much broader than that of *I. persulcatus*, suggesting that residents of a much broader area of Kazakhstan may be at risk for exposure to the virus (14). We are currently investigating the role of raw milk and cheese in the spread of TBE.

In summary, we have substantial new data on a serious tick-borne disease in Central Asia, of importance both to local and global public health authorities, as well as the US DoD. Acknowledging these successes, and the essential collaboration of the Kazakhstani and Kyrgyz authorities and our scientific colleagues, there are nevertheless issues that impede further development of our novel findings; these issues are discussed in the Section "Conclusion: Accomplishments and Challenges" below.

RICKETTSIAL DISEASES

Historically, tick-borne rickettsial diseases in Kazakhstan were attributed solely to Siberian tick typhus. Siberian tick typhus is caused by *Rickettsia sibirica* subsp. *sibirica* that is transmitted by ixodid ticks (i.e., *Dermacentor* and *Haemaphysalis* spp.) (18). Between 2007 and 2012, 1,247 registered cases of Siberian tick typhus were recorded in Kazakhstan, with the highest prevalence (65%) occurring Kyzylorda and North Kazakhstan Oblasts (17). Whether these cases were truly due to Siberian tick typhus and/or other rickettsioses was not clear, because serological assays are cross-reactive for antibodies

[2]http://www.vectormap.org/dataportal.htm

against the spotted fever group rickettsiae (SFGR) (19). Indeed, recent tick surveys have identified four additional SFGR species that may be responsible for rickettsial diseases in Kazakhstan (18, 20–22). These four agents, *Rickettsia conorii* subsp. *caspia*, *Rickettsia slovaca*, *Rickettsia raoultii*, and a *Rickettsia aeschlimannii*-like organism, were identified in collaborative studies between Russian and Kazakhstani scientists (18, 20–22).

Additional studies performed in Kazakhstan confirmed the presence of tick-borne rickettsiae among ticks collected through tick drags and small mammal trapping. During the spring of 2004, a study conducted by the UAPS in the southern tip of West Kazakhstan Oblast collected 33 *Rhipicephalus pumilio* ticks. Two ticks (6%) were positive for rickettsial DNA and data from subsequent sequencing analyses identified the agents as *R. conorii* subsp. *caspia*, the causative agent of Astrakhan spotted fever (17). Assessment of 330 ticks collected by tick drag in Karaganda Oblast in Central Kazakhstan by SPCSEEM determined that they were all *D. marginatus*. Of the 33 pools of these ticks tested for infection by rickettsial pathogens (10 ticks per pool), 2 from the Abai Rayon were positive (17).

Most recently (2012–2014), one large tick surveillance study supported by US Defense Threat Reduction Agency (DTRA) was conducted in Kazakhstan. One aspect of the investigation focused on detecting tick-borne rickettsiae and involved ticks collected in seven oblasts: Atyrau, East Kazakhstan, Zhambyl, Karaganda, Kyzylorda, Pavlodar, and West Kazakhstan involving SPCSEEM staff in Almaty (17) and UAPS staff in Uralsk (17, 23). The project explored both the identification of tick species and the detection of *Rickettsia* positive ticks by qPCR and multilocus sequencing typing (MLST); data analysis is ongoing.

Q FEVER

Q fever is caused by the Gram-negative bacterium, *Coxiella burnetii*, and is found all over the world (24). More than 40 species of ticks are known to be naturally infected with *C. burnetii*, and though tick-to-human transmission does occur it is only thought to be responsible for a small subset of infections (25). The most common route of human infection is through the inhalation of aerosolized particles, generally the result of the aerosolization of dried parturition materials from infected animals (26). Infected animals also shed *C. burnetii* in urine, feces, and milk, the last of which may cause infection when consumed without pasteurization (25). Q fever has been reported in Kazakhstan since the early 1950s (27). Currently, UAPS is conducting a study supported by DTRA assessing the presence of *C. burnetii* in unpasteurized milk in the West Kazakhstan Oblast utilizing a qPCR assay targeting IS1111 DNA (28).

CONCLUSION: ACCOMPLISHMENTS AND CHALLENGES

Accomplishments

During the last decade in Kazakhstan and Kyrgyzstan, we have carried out extensive training and research work in collaboration with our local colleagues. These efforts have led to enhanced biosafety and biosecurity practices, successful implementation of field and laboratory techniques, and awareness and compliance with regulatory standards. Modern rodent capture methods were also introduced, which included training on the use of live capture traps and safe rodent handling. As a result, we have been able to develop technical presentations in a number of research areas, many of which are cited in the reference list.

Our collaborative studies have also generated extensive collections of field samples and associated analytical datasets. For example, we now have over 40,000 ectoparasites from tick drags and vertebrate hosts, many of which have been identified both by morphological and molecular means. Data, including tick species, location (GPS coordinates), and date of collection were recorded and shared via public databases.

In the laboratory, enhanced sample processing and use of molecular diagnostics were emphasized. Safe and proper procedures for bead-beating tick samples were employed, and nucleic acid extraction (DNA and RNA) was performed on those samples before qPCR determination of bacterial and viral identity. We also demonstrated and implemented methods that comply with Federal Wide Registration (FWA) standards for testing of stored human sera.

Finally, between 2011 and 2014, our work in central Asia produced 20 scientific papers presented at 11 professional conferences. These presentations were developed in collaboration with our in-country colleagues. In addition, one peer-reviewed manuscript based on this work has been published to date (8).

Overall, these considerable accomplishments have resulted in a better trained and aware scientific workforce in Kazakhstan and Kyrgyzstan. Our central Asian colleagues have been exposed to modern scientific methodology and perspectives and have been able to present their work in several major international forums. This has led to significant findings in public health in this part of Central Asia, which will increase awareness of important infections locally and globally. Finally, this work will provide a solid basis for future planning for infectious disease surveillance and research in Central Asia.

Challenges

In the course of all the successful work described above, we encountered a number of challenges that are being addressed to facilitate continued progress in Kazakhstan and Kyrgyzstan. From the literature, one can see that only a few surveyed locations in this region are reported in peer-reviewed international journals. Even from the last large study that surveyed many oblasts, the number of rayons (i.e., county or district) studied within each oblast was limited, and thus the overall investigation is constrained. An obvious solution would be to continue and expand biosurveillance studies, varying the sites over time, but is time consuming and expensive.

Another issue is the limited submission of research results to peer-review, high impact international journals, especially English language journals, by local scientists. Scientists around the world are developing the scientific credentials through successful submission of their results to peer-review journals. This

submission process needs to be encouraged for scientists in Central Asia, especially in regards to writing detailed results and statistics required for publication in peer-reviewed, international journals. In addition, the development of this writing skill and the publication of notable results will enhance the success at developing and submitting proposals for local and international grant funding.

A further crucial area for improvement is the development of technical skills for assessing and developing assays and methods, as well as data analysis. Lack of standard operating procedures in Russian, Kazakh, and Kyrgyz languages, and trained technical personnel are other problems. Current reliance on commercial test assays is costly and unsustainable. Possible solutions to these problems include developing reagent/equipment procurement streams via known in-country and/or regional commercial entities and developing in-house assays that can be compared with currently accepted commercial assays. Increasing molecular diagnostics capabilities at institutions, including government, university, and commercial entities, would also be useful, particularly if these could be shared with among institutions. Limited submission of samples for analysis and verification to outside institutions lessens future progress. This issue can only be solved by allowing scientists to bring or send test sample material to foreign institution; this will not be simple to resolve and will require substantial political will on the part of the local authorities.

Finally, there are many communication barriers to overcome. This would be helped by including each level of work group (including technicians, scientists, managers, and directors) in discussions of research activity, as well as encouraging clear communication within organizations to improve research and planning. In our work, we found that direct communication, especially face-to-face meetings and conference calls, was most effective and decreased the need for written correspondence that required language translation.

Overall, the accomplishments in the biosurveillance studies conducted in Kazakhstan and Kyrgyzstan presented here demonstrates that significant work can be achieved despite existing challenges. With time and continued collaborative efforts, we believe that these challenges will overcome, leading to even more progress in the future. Current interest in global health security activities coupled with the historic zoonotic diseases in this part of the world provides ample opportunities for further studies.

ACKNOWLEDGMENTS

We gratefully recognize the United Kingdom Ministry of Defence Biological Engagement Programme, US Defense Threat Reduction Agency, and the US Department of State for funding the research described in this review. The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Navy, Department of Defense, or the United States Government. This is the work of US government employees (AR and CF) work unit number A1266 and may not be copyrighted (17 USC 105). We extend special thanks to Kavita Berger, Charles Buck, and Barry Atkinson for their invaluable insights and suggestions in developing this manuscript.

REFERENCES

1. McNamara T, Platonov A, Elleman T, Gresham L. The human-animal interface and zoonotic threats: the Russian Federation approach. *Biosecur Bioterror* (2013) **11**(93):185–95. doi:10.1089/bsp.2013.0054
2. Ouagrham-Gormley S. Growth of the anti-plague system during the soviet period. *Crit Rev Microbiol* (2006) **32**:33–46. doi:10.1080/10408410500496789
3. Zilinskas R. The anti-plague system and the Soviet biological warfare program. *Crit Rev Microbiol* (2006) **32**:47–64. doi:10.1080/10408410500496896
4. Ouagrham-Gormley SB, Melikishvili A, Zilinskas RA. *The Anti-Plague System in the Newly Independent States, 1992 and Onwards: Assessing Proliferation Risks and Potential for Enhanced Public Health in Central Asia and the Caucasus*. Monterey, CA: James Martin Center for Nonproliferation Studies, Monterey Institute of International Studies (2008).
5. National Research Council. *Science and Technology in Kazakhstan: Current Status and Future Prospects*. Washington, DC: The National Academies Press (2007).
6. Hotez PJ, Alibek K. Central Asia's hidden burden of neglected tropical diseases. *PLoS Negl Trop Dis* (2011) **5**(9):e1224. doi:10.1371/journal.pntd.0001224
7. Han BA, Schmidt JP, Bowden SE, Drake JM. Rodent reservoirs of future zoonotic diseases. *Proc Nat Acad Sci U S A* (2015) **112**:7039–44. doi:10.1073/pnas.1501598112
8. Nurmakhanov T, Sansyzbayev Y, Atshabar B, Kazakov S, Derjabin P, Kazakov S, et al. Crimean-Congo hemorrhagic fever virus in Kazakhstan, 1948-2013. *Int J Infect Dis* (2015) **38**:e19–23. doi:10.1016/j.ijid.2015.07.007
9. Program and Abstracts Book. *11th ASM Biodefense and Emerging Diseases Research Meeting*. Washington, DC: ASM Press (2013). p. 50.
10. Mansfield KL, Johnson N, Phipps LP, Stephenson JR, Fooks AR, Solomon T. Tick-borne encephalitis virus – a review of an emerging zoonosis. *J Gen Virol* (2009) **90**:1781–94. doi:10.1099/vir.0.011437-0
11. Bogovic P, Strle F. Tick-borne encephalitis: a review of epidemiology, clinical characteristics and management. *World J Clin Cases* (2015) **16**:430–41. doi:10.12998/wjcc.v3.i5.430
12. Lvov DR, Shchelkanov MY, Alkovsky SV, Deryabin PG. *Zoonotic Viruses of Northern Eurasaia: Taxonomy and Ecology*. London: Academic Press (2015).
13. Briggs B, Atkinson B, Czechowski D, Larsen P, Meeks H, Carrera J. Tick-borne encephalitis Kyrgyzstan. *Emerg Infect Dis* (2011) **17**:876–9. doi:10.3201/eid1703.101183
14. Final Program ASM 2014. American Society for Microbiology. *114th General Meeting*. Boston, MA; Washington, DC: ASM Press (2014). p. 182.
15. Zlobin VI, Verkhozina MM, Demina TV, Dzhioev IP, Adel'shin RV, Kozlova IV, et al. Molecular epidemiology of tick-borne encephalitis. *Vopr Virusol* (2007) **52**(6):4–13.
16. Final Program ASM 2014. American Society for Microbiology. *114th General Meeting*. Boston, MA; Washington, DC: ASM Press (2014). p. 192.
17. Final Program ASM 2014. American Society for Microbiology. *114th General Meeting*. Boston, MA; Washington, DC: ASM Press (2014). p. 139.
18. Rudakov NV, Shpynov SN, Samoilenko IE, Tankibaev MA. Ecology and epidemiology of spotted fever group rickettsiae and new data from their study in Russia and Kazakhstan. *Ann N Y Acad Sci* (2003) **990**:12–24. doi:10.1111/j.1749-6632.2003.tb07332.x
19. Luce-Fedrow A, Mullins K, Jiang J, Richards AL. Strategies for detecting rickettsiae and diagnosing rickettsial diseases. *Future Microbiol* (2015) **10**:537–64. doi:10.2217/fmb.14.141
20. Shpynov SN, Parola P, Rudakov NV, Samopenko IE, Tankibaev MA, Tarasevich I, et al. Genotyping of spotted tick fever rickettsiae, detected in Russia and Kazakhstan. *Med Parazitol (Mosk)* (2003) **3**:20–4.
21. Shpynov S, Fournier PE, Rudakov N, Tankibaev M, Tarasevich I, Raoult D. Detection of a *Rickettsia* closely related to *Rickettsia aeschlimannii*, "*Rickettsia heilongjiangensis*," *Rickettsia* sp. strain RpA4, and *Ehrlichia muris* in ticks

collected in Russia and Kazakhstan. *J Clin Microbiol* (2004) **42**:2221–3. doi:10.1128/JCM.42.5.2221-2223.2004
22. Samoylenko I, Shpynov S, Raoult D, Rudakov N, Founier PE. Evaluation of *Dermacentor* species naturally infected with *Rickettsia raoultii*. *Clin Microbiol Infect* (2009) **15**:305–6. doi:10.1111/j.1469-0691.2008.02249.x
23. Petersen E, Barkham T, Brown S, Gonzalez R, Leblebicioglu H, Hoepelman AIM, et al., editors. International journal of infectious diseases. *16th International Congress on Infectious Diseases (ICID)*. Cape Town: Elsevier (2014). p. 393.
24. Oyston PCF, Davies C. Q fever: the neglected biothreat agent. *J Med Micrbiol* (2011) **60**:9–21. doi:10.1099/jmm.0.024778-0
25. Maurin M, Raoult D. Q fever. *Clin Microbiol Rev* (1999) **12**:518–33.
26. Huebner RJ, Jellison WL, Beck MD, Parker RR. Q fever studies in Southern California. I. Recovery of *Rickettsia burnetii* from raw milk. *Public Health Rep* (1948) **63**:214–22. doi:10.2307/4586445
27. Tsoi DC, Rapoport LP, Samartseva ET. Epidemiological studies of Q fever in the area of Dzhambal in the Kazakh SSR. *J Hyg Epidemiol Microbiol Immunol* (1980) **24**:206–11.
28. Jones RM, Twomey DF, Hannon S, Errington J, Pritchard GC, Sawyer J. Detection of *Coxiella burnetii* in placenta and abortion samples from British ruminants using real-time PCR. *Vet Rec* (2010) **167**:965–7. doi:10.1136/vr.c4040

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The Reviewer Brad S. Schneider and handling Editor Nathan Wolfe declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Public Health

ORIGINAL RESEARCH
published: 24 March 2016
doi: 10.3389/fpubh.2016.00044

Biosafety Initiatives in BMENA Region: Identification of Gaps and Advances

Erum Khan[1], Nayla Ahmed[1], Khalid R. Temsamani[2], Atef El-Gendy[3], Murray Cohen[4], Ariba Hasan[1], Hilliard Gastfriend[4] and Jennifer Cole[4]*

[1] Aga Khan University Karachi, Karachi, Pakistan, [2] Faculté des Sciences de Tétouan Mhannech II, University Abdelmalek Essaadi, Tétouan, Morocco, [3] Bacteriology Section, Naval Medical Research Unit 3 (NAMRU-3), Cairo, Egypt, [4] Frontline Foundation, Atlanta, GA, USA

Introduction: The objectives of this study were to identify and assess the impact of capacity-building biosafety initiatives and programs that have taken place in the broader Middle East and North Africa (BMENA) region between 2001 and 2013, to highlight gaps that require further development, and to suggest sustainable ways to build cooperative regional biosafety opportunities.

Methods: A cross-sectional study was conducted with two aspects (1) thorough desktop review of literature for all biosafety/biosecurity-related activities in the study countries, such as seminars, conferences, workshops, policy documents, technology transfer, sustained scientific endeavors between countries, etc. and (2) an online survey of scientists in countries in the region to get first-hand information about biosafety and biosecurity initiatives and gaps in their country.

Results: A total of 1832 initiatives of biosafety/biosecurity were recorded from 97 web links; 70.68% (n = 1295) initiatives were focused on raising general awareness among the scientific community about biosafety/biosecurity/biocontainment. The most frequent areas of interest were biorisk management in biomedical and biotechnology laboratories 13% (n = 239), followed by living modified organisms (LMOs) 9.17% (n = 168). Hands-on training accounted for 2.67% (n = 49) of initiatives. Online survey results confirmed desktop review findings; however, the response rate was 11%.

Keywords: biosafety, biosecurity code of conduct, survey research, BMENA region, biorisk management

OPEN ACCESS

Edited by:
Jeanne Marie Fair, Defense Threat Reduction Agency, USA

Reviewed by:
Tim Trevan, Chrome Biorisk Management, USA
Maureen O. Leary, Dartmouth College, USA

****Correspondence:***
Erum Khan
erum.khan@aku.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *06 August 2015*
Accepted: *04 March 2016*
Published: *24 March 2016*

Citation:
Khan E, Ahmed N, Temsamani KR, El-Gendy A, Cohen M, Hasan A, Gastfriend H and Cole J (2016) Biosafety Initiatives in BMENA Region: Identification of Gaps and Advances. Front. Public Health 4:44. doi: 10.3389/fpubh.2016.00044

INTRODUCTION

Recent advances in biotechnology have provided a quantum leap in the application of biological sciences in all fields, including health, agriculture, environment, and energy development. Biotechnology tools and protocols are globally available and are increasingly being used and seen as potential investments. However, with rapid advancement, widespread use, and investment, comes the possibility of exploitation of research and risk of misuse of the research outcomes for nefarious purposes. The most concerning issue remains the lack of expertise and awareness of risk management systems for the mitigation of such risks in the biological sciences, despite extensive

and well-developed relevant engineering and scientific methods for containment, detection, diagnosis, treatment, and recovery as well as human behavior and performance science in other fields, such as aviation, nuclear power, petrochemicals, etc. This concern has been raised globally and has intensified since the events of 9/11 and the anthrax-in-the-mail terrorism of 2001. This has led to international focus on means for combating bioterrorism, especially in countries with the backdrop of disturbed geopolitical situations (1–5).

This study was undertaken to identify and assess the impact of capacity-building biosafety initiatives and programs that have taken place in broader Middle East and North Africa (BMENA) region between 2001 and 2013, and to highlight gaps that require further development. The project had two components: (a) a review of biosafety and biosecurity initiatives in the region and (b) assessment of the impact of these initiatives on scientists at local/regional levels. This study was part of a larger project titled "*Scientific Engagement Defining Gaps and Creating Opportunities for Cooperative Research and Global Security in the Broader Middle East and North Africa Region*" to assess the overall impact of global biosecurity capacity-building initiatives undertaken in recent years in the BMENA region and to then apply the knowledge gained from this assessment to suggest sustainable ways to build cooperative regional biosafety opportunities.

MATERIALS AND METHODS

The project co-PIs, based in Pakistan and the U.S., worked closely with two Regional Coordinators in North Africa (Morocco and Egypt) to recruit a project coordinator in each of the 24 countries participating in the project. A cross-sectional descriptive study was designed consisting of two steps: (1) a desktop review/literature search for all biosafety- and biosecurity-related initiatives that were reported from the BMENA region between 2001 and 2013 using commercial search engines and (2) building a survey instrument in the commercially available SurveyMonkey (SM) platform and administering it independently to scientists in the study countries.

A database was created according to the type of initiatives each study country in the region had, the scope of the activity, institute(s) involved, and type of funding/donors, etc. Great care was taken to develop a sampling process that would protect the confidentiality of survey participants and their responses. A cross-sectional online survey was conducted throughout the region. The survey questions sought to collect broad observations about overall capacity needs or gaps in biorisk management systems in the BMENA study countries. It included questions about technically skilled human resources in various aspects of biosafety management systems, availability of sustainable training programs, and challenges such as lack of human resources, monetary funds, specific bio risk management skills, etc. Questions were also developed to assess capacity for biorisk management and oversight regulation, such as availability of national/institutional biosafety committees and scientist/professionals for regulatory compliance assistance. Institutional Review Board approval was obtained through the Aga Khan University in Karachi, Pakistan. Survey subjects were identified by the country coordinators.

Subject Inclusion Criteria

The following member countries as per World Health Organization (WHO) definition of BMENA region were included in the study: Afghanistan, Algeria, Bahrain, Egypt, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Libya, Mauritania, Morocco, Oman, Palestine, Pakistan, Qatar, Saudi, Sudan, Syria, Tunisia, Turkey, United Arab Emirates (UAE), and Yemen. Members of biosafety associations of these countries were invited by the study country coordinators to participate in the survey. In countries without biosafety associations, members of other scientifically related associations were invited. Thirty members of these various associations from each country were identified by random selection to receive the survey questionnaire *via* email.

Analysis

Data from desktop review were entered and analyzed using Microsoft Excel (Windows XP 2008). Results were analyzed as categorical data. The biosafety initiatives reported from member countries were categorized as per the following headings: (a) general awareness sessions: activity conducted to raise general knowledge/education of biosafety and biosecurity among the scientific community; (b) human resource development: activity that translated into hands-on training of the scientists in a particular aspect of biosafety and biosecurity with an aim to transfer technology and to provide expertise at local or regional levels; (c) institutional capacity building: activity that resulted in development of network/association/foundation that would foster the biosafety and biosecurity development at the local/regional levels; (d) scientific collaborations between the member countries or with international communities which included initiatives such as regional conferences/meetings; and (e) sustainable collaborative projects at regional levels such as infectious disease surveillance, formal laboratory biosafety and biosecurity training, responsible science/bioethics and scientific cooperation, student exchange, etc. Additional information where available was recorded regarding the duration of activity, funding support, and feedback from participants of the activity.

The survey was kept open for a duration of 10 working days, and responses were directly downloaded from SM and analyzed/graphed in Microsoft Excel. The survey results were analyzed as ordinal data, responses to various survey questions were recorded as ordered categories (excellent to poor). Frequencies of the responses were generated as percentages of excellent, good, average, and poor categories of resource availability.

RESULTS

Desktop Review

A total of 1832 biosafety/biosecurity initiatives were recorded from 97 web links that were reviewed, after removing the duplicate, 60 web links (1–60) were used for analysis. It was encouraging to note that every country listed in the BMENA region had some initiatives related to biosafety/biosecurity between 2001 and 2013 (**Figure 1**). The focus of these initiatives was found to be general awareness in the field of biotechnology and biomedical laboratories with particular interest in risk assessment and

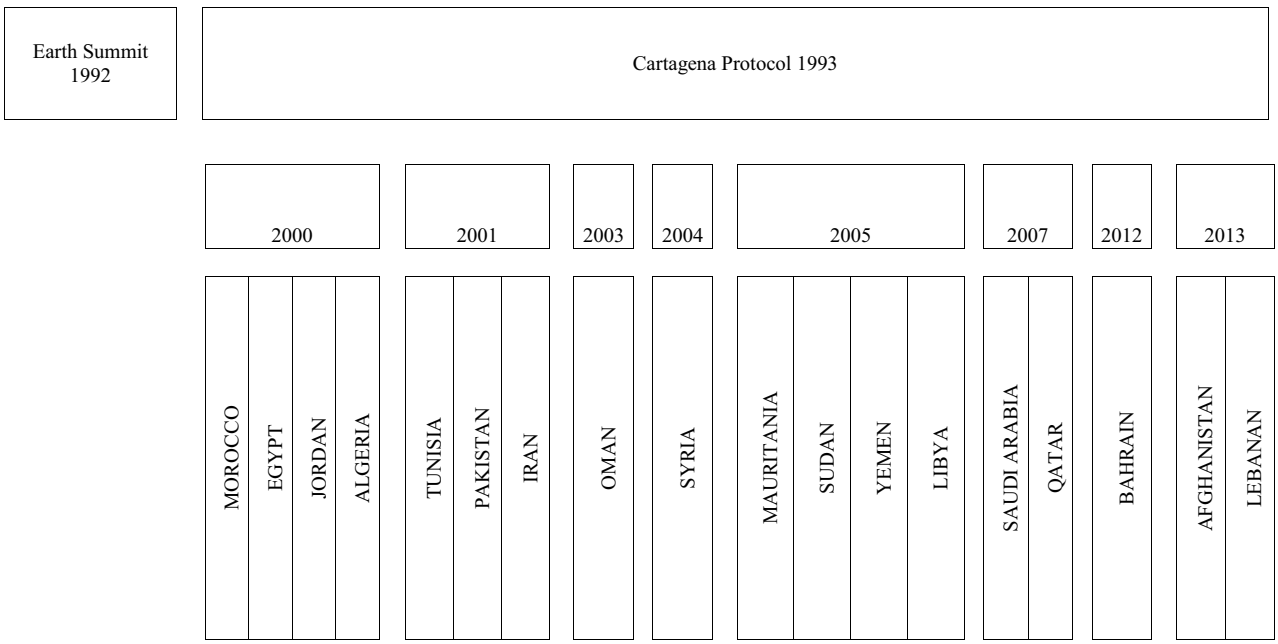

FIGURE 1 | Time lines for the biosafety initiatives in BMENA region 2001–2013. Time lines of biosafety and biosecurity initiatives in BMENA region following Cartagena Protocol. The boxes represent year in chronological order in which biosafety initiative could be traced for the member countries by desktop review.

mitigation 13% ($n = 239$), followed by awareness of genetically modified organisms (GMOs) 9.17% ($n = 168$). Very little data were available for initiatives for other areas of significant biosafety concern, such as animal health sciences, chemical industry, or radiological industry.

When categorized into types, most of the initiatives were found to be in the category of general awareness and information exchange workshops at country level 70.68% ($n = 1295$). The topics most commonly addressed in these awareness sessions included basic biosafety, biosecurity, biocontainment, and biorisk management in biomedical and biotechnology laboratories (6–39). Other aspects included bioterrorism and means to combat it, as well as bioethics and dual use resebiosafety, biosecurity, biocontainment, and biorisk management in biomedical and biotechnology laboratories (6–39). Other aspects included bioterrorism and means to combat it, as well as bioethics and dual use research of concern and other ethical dilemmas (21, 34, 38, 40–44). Scientific conferences and meetings on GMOs and other biotechnology-related topics (31, 45–49) were the next most frequently conducted initiatives at 18.5% ($n = 339$) (see **Figure 2**).

Capacity building was defined for this project as any initiative focused on technology transfer, hands-on training, and assistance in development of national framework, i.e., development of national/institutional biosafety committees or sustainable scientific projects with common regional issues and long-term measureable outcome, such as infectious disease surveillance/laboratory diagnosis, etc., in the region (**Figure 3**). Of the initiatives reviewed, 2.67% ($n = 49$) were in the category of hands-on training. Very few initiatives were identified using key words "student exchange program, regional training centers, and disease surveillance" against collective term BMENA, representing the region (50, 51).

An increased international donor interest was noted in the BMENA region for biosafety and biosecurity initiatives post 2000; 80% of the initiatives reviewed were funded by international donor/scientific agencies such as the United Nations WHO and the Environment Programme (UNEP), the International Council for Life Scientists (ICLS), and CRDF Global. United States sponsored/funded biosafety activities organized in 2001–2013 in the BMENA region included the Department of State Cooperative Biosafety Engagement Program (CBEP) and the American Association for the Advancement of Science-Center for Science, Technology, and Security Policy (AAAS-CSTSP).

Jordan appeared to be the hub in the region for biosafety, biosecurity, and biorisk management activities with ($n = 179$), followed by Pakistan ($n = 162$), Morocco ($n = 109$), Iraq ($n = 68$), Iran ($n = 66$), and Egypt ($n = 60$). This involved organization and hosting of national and international conferences, seminars, and training workshops on general awareness aspects of biosafety and biosecurity (**Table 1**).

Online Survey

The survey was administered to the life scientists identified for each participating country using the commercially available MailChimp software program within the SM platform, an internationally recognized leader in online surveying technology. SM offers state-of-the-art features in both survey design and delivery capabilities. In order to help manage panels of potential survey respondents, SM has developed a dedicated email service called MailChimp, which allows for targeted delivery of survey requests for participation.

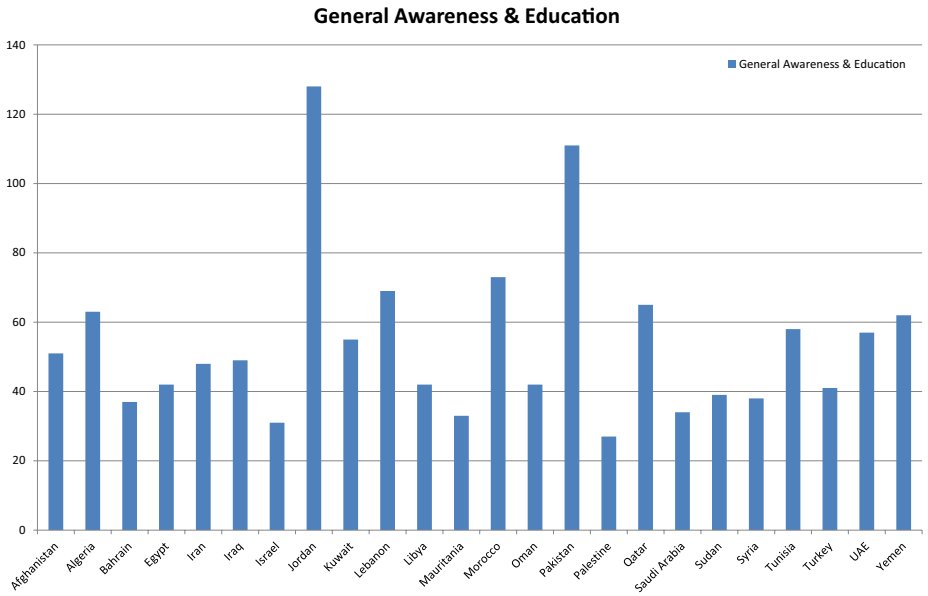

FIGURE 2 | General awareness and educational initiatives BMENA region 2001–2013. General awareness initiatives were defined as an activity conducted to impart general knowledge of biosafety and biosecurity among the scientific community to raise general awareness. Most of these activities were conducted in form of workshops and seminars.

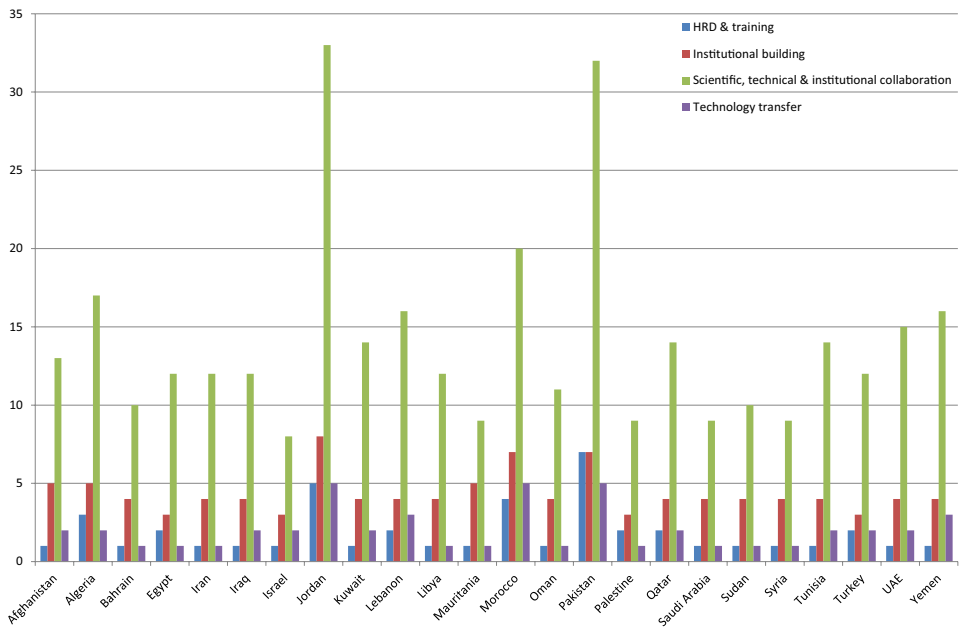

FIGURE 3 | Other biosafety initiatives in BMENA region conducted during study period 2001–2013.

The survey was initially released as a pilot, using Pakistan as the test group. The pilot survey was released on June 5, 2013: 50 requests to participate were sent out, with 25 participants opening the request, and 23 agreeing to participate. As per the IRB protocol, respondents were required to agree to take the survey once they understood any potential harm by participating before they could proceed to the questions within. Thus, they could open the survey, agree to the human ethics considerations, and then view the remaining questions. At this point, most respondents stopped participating. A disclaimer was introduced at this point stating that all data will be confidential and anonymous.

The final survey was released on June 30, 2013. Approximately 215 survey invitations were sent out among 11 countries (some country associations had less than 30 members). Twenty-eight recipients reviewed the survey, and 23 agreed to participate. This 11% participation rate was less than desired, although we expected a high response rate to be difficult from the countries in this study. Generally online survey responses would be expected to be in the 15–20% range, but cultural differences, possible fear of responding (despite statements declaring that all data were confidential and anonymous), and lack of experience with this type of information gathering might account for the lower rate. As a result, hard copies were posted to the country coordinators to send to their colleagues, but the response rate to the requested hard copies survey was also very poor. Iraq, Afghanistan, Sudan, Yemen, Algeria, and Syria had requested hard copies so that potential respondents would have access to all survey questions to determine if the survey was too dangerous or risky for participation. In the main release, 18% of respondents viewed the survey but did not answer the questions.

TABLE 1 | Country list of institutes/organizations that conducted or collaborated toward biosafety and biosecurity initiatives in the BMENA region 2001–2013.

No	Country	Organizations/institutes
1	Afghanistan	Afghan Biorisk Association
2	Jordan	El Hassan Science City (EHSC)/Royal Scientific Society (RSS) Middle East Scientific Institute For Security (MESIS) [earlier known as Cooperative Monitoring Centre (CMC)] Higher Council for Science and Technology Jordan University of Science and Technology/Princess Haya Biotechnology Centre University of Jordan/Hamdi Mango Center for Scientific Research (HMCSR)
3	Lebanon	Lebanese Agricultural Research Institute American University of Beirut Lebanese National Council for Scientific Research
4	Libya	Environment General Authority of Libya Libyan Association for Biotechnology Libyan National Committee for Bioethics, Biosafety and Biosecurity
5	Morocco	Moroccan Biosafety Association
6	Tunisia	Ministry of Higher Education and Scientific Research in Tunisia Centre of Biotechnology of Sfax Tunisia The Tunisian Association of Biotechnology
7	Pakistan	Pakistan Biological Safety Association Biological Safety Association of Pakistan National Task Force for Biosafety initiatives
8	Israel	Israel Biological Safety Association
9	Turkey	Biotechnology Association of Turkey Biosafety and Bio Economy Association of Turkey
10	Iran	Iranian Biosafety Association
11	Egypt	Egyptian Biological Safety Association

Although the response rate was lower than desired, the respondents were people with relevant background. Fifty-two percent of the respondents had worked as laboratory directors/managers in their fields and 70% of these were directly involved in biorisk management activities of their institutes. The survey results are reflective of the desktop review. Seventy-one percent of the respondents marked yes to the question about initiatives on general awareness on biosafety and biosecurity in their country. Of the respondents, 52% felt that biosafety initiatives in their countries were organized by NGOs, including professional/scientific societies, while 23.8% believed they were organized by the government ministries in their country. Fifty-two percent responded in the negative to the questions asking if the international initiatives were demand driven and sustainable.

Table 2 shows the results of direct questions related to the availability and reliability of different technical skills for biorisk management in the respective countries, on the scales of excellent to poor. The scale was defined as *excellent* where the technical expertise was easily available and considered highly reliable by the local scientists; *good* where technical expertise was available but was not very actively sought by local scientists (i.e., marginally reliable); *average* was defined as very limited or scarce local

TABLE 2 | Survey results of respondents from 11 member countries of BMENA region 2013–2014.

	Excellent	Good	Average	Poor
Availability of technically skilled laboratory workers	22.5	42.5	30.0	7.5
Availability of skilled biosafety professionals or biorisk managers	10.0	22.5	27.5	40.0
Availability of scientists skilled in risk assessment for biohazards	2.5	20.0	40.0	32.5
Availability of technicians skilled in overseeing effective engineering controls (HVAC, BSC, etc.)	10.0	20.0	27.5	37.5
Availability of technically skilled professionals to oversee laboratory design	5.0	20.0	22.5	40.0
Availability of technically skilled workers for laboratory operation and maintenance	10.0	27.5	25.0	32.5
Availability of technically skilled workers for handling/transfer of GMO	5.0	17.5	22.5	50.0
Availability of technically skilled workers for handling/transfer of potentially infectious material	7.5	17.5	42.5	32.5
Availability of technically skilled animal handling workers	5.0	25.0	27.5	32.5
Availability of technically skilled workers with blood-borne pathogens	10.0	30.0	30.0	27.5
Availability of infrastructure and professional staff to implement biorisk management programs, including SOPs	10.0	22.5	22.5	37.5
Availability of accredited biorisk management training for senior scientists	5.0	7.5	20.0	55.0
Availability of accredited biorisk management training for lab directors or managers	5.0	12.5	15.0	60.0
Availability of accredited biorisk management training for university and graduate students	2.5	7.5	30.0	55.0
Availability of biorisk management training/teaching resources and materials	7.5	17.5	25.0	42.5
Availability of national/institutional biorisk management oversight, such as regulatory compliance assistance, or institutional biorisk management committees	7.5	17.5	17.5	47.5

expertise and that was not very credible (i.e., marginally reliable); and unavailability of any local expertise was rated as *poor*.

Survey results were in accordance with the desktop review findings of biosafety initiatives during the 2001–2013 time period. We found initiatives to be largely focused on general awareness or on introductory courses on biosafety with limited human resource training and technology transfer opportunities. Consequently, there was a dearth of skilled human resources in the region as evident from survey results.

DISCUSSION AND RECOMMENDATIONS

Significant advancements in the field of biotechnology in the late 1990s and early 2000s raised international concerns for biosafety related to biodiversity, particularly pertaining to risk assessment and risk mitigation regarding the impacts of new research products. These concerns were more pronounced in the agriculture field with development of Bt. cotton (GMOs) and its impacts on natural plants and the environment. These international concerns, at the forefront in the United Nations Conference on Environment and Development (UNCED), were formally recognized in what became the first Earth Summit, held in Rio de Janeiro in June 1992 (1, 2). This resulted in the Convention on Biological Diversity, from which originated the 1993 Cartagena Protocol of Biosafety. The terms biosafety and biosecurity, thereafter, gained much popularity and focus among the scientists and policy makers globally. In the BMENA region, biosafety-related initiatives can be traced back to as early as the year 2000 (1), as shown in **Figure 1**. We found the Cartagena protocol to be a catalyst for the serious initiatives in the capacity-building efforts in the BMENA region. However, most of the early initiatives were focused on national frameworks for biotechnology, aspects such as control GMOs and living modified organisms (LMOs), and environmental effects of agricultural products, such as Bt Cotton, perhaps because of its wider impact at the global level (2). We found 18% of total initiatives to be focused on biosafety issues related to LMOs. Although these are encouraging figures, the BMENA region is the most water-scarce and dry region worldwide. Countries across the region, especially those around the Mediterranean Sea which are highly dependent on agriculture, are tempted to use GMOs to meet consumer food demand. Therefore, more concentrated efforts are required to initiate open forums to discuss current controversies related to the pros and cons of the use of LMOs and GMOs, as well as the long-term effects on local biodiversity. The future of genetically engineered foods and crops in BMENA region will depend heavily on choices governments make regarding the regulation of this technology; therefore, coordinated and strong regional efforts are urgently required. The BMENA Organization for Economic Co-operation and Development (OECD), a forum in which BMENA governments work together to share experiences and seek solutions to common economic problems, can perhaps be an effective forum to raise the concern about the local legislations, uses, and transportation of LMOs in the region.

Post 9/11 and anthrax in the mail bioterrorism in 2001, the focus on biosafety initiatives in the BMENA region broadened to include other sciences, mainly biomedical sciences and to some extent veterinary sciences, bioengineering, chemical/nuclear sciences, and agricultural sciences, especially in countries with the backdrop of dynamic or unstable geopolitical circumstances (46–49). With the support of international organizations that focus on biosafety and biosecurity, a number of biosafety initiatives took place after 2000 in the scientific community of the BMENA region. These initiatives were primarily focused on raising awareness about biosafety and biosecurity.

Seventy percent of the total initiatives recorded fell in the category of general biosafety awareness sessions, which is an encouraging finding. However, interpreting these results at the country level is most challenging and complicated because of the diversity of the population strata, cultures, socioeconomic statuses, availability of funds, and development needs of study countries. For example, disparity is noticeable in terms of number of initiatives when compared with the per capita population of member countries. The 162 initiatives in Pakistan, the sixth most populous country in the world with an estimated population of 184.35 million, was desperately low as compared to 109 initiatives in the country of Morocco and 62 for Qatar with populations of 32 and 2.27 million, respectively. Thus, more sophisticated studies are required at individual country level for representative situational analysis.

Capacity-building activities were difficult to assess, as it is more of a conceptual approach and varies country to country depending upon needs to successfully execute the biosafety and biosecurity activities. For this, project capacity building was defined as any initiative that was focused on technology transfer, hands-on training, and assistance in development of national frameworks or sustainable scientific projects, with common regional issues and long-term measurable outcomes such as infectious disease surveillance/laboratory diagnosis. Initiatives to develop local expertise by providing hands-on training to professionals in the region were found to be significantly lacking during 2001–2013; this was also evident from the findings of the online survey. The majority of the responses to the direct questions in the survey about the availability of reliable expertise in various technical components of biorisk management were rated as average to poor. Thus, success of sustainable biosafety progress in the region demands strengthening the expert human resource training to provide the region with a wider group of local experts with sound skills in biorisk management. Such a group of skilled professionals would then be able to create guidelines and standards uniquely suited to their circumstances. Such efforts would foster a sense of ownership of guidelines; local solutions to local problems would raise the confidence and reliance on local experts by the regional scientific community.

The noticeable general trend was the international donor interest in the BMENA region in 2001–2013. Multiple donor agencies from around the globe either funded or collaborated with the biosafety initiatives in the region. However, we found that most of the efforts had been at the individual country level and not at a regional level, resulting in some duplication of efforts. For example, six separate UNEP-funded biosafety and biosecurity awareness initiatives were recorded from individual countries between January 2002 and December 2003, including Algeria, Iraq, Iran, Turkey, Egypt, and Syria. Such duplication can best be avoided in the future by working more closely with regional groups in order

to coordinate efforts and focus on regional needs. Moreover, international donor funded activities related to human resource training in some study countries were coordinated through local government authorities, such as the Ministry of Agriculture of Lebanon, the Ministry of Regional Municipalities Environment and Water Resources of Oman, and the National task force in Pakistan. Successful completion of such projects was found to be dependent on political relationships between the donor and recipient country, and was often subjected to premature termination (personal communications with the country collaborators).

Another trend noticeable from the desktop review was that countries with actively functional NGOs working as Biosafety Associations, and/or those with institutes with biosafety missions, had far more initiatives on general awareness of biosafety-related issues than those without such associations. These countries hosted biosafety programs, reflecting the heightened concern in the region. Future initiatives fostering private–public partnership are strongly recommended for successful and sustainable outcomes. Successful public/private partnership models that currently exist include the International Council for the Life Sciences (a U.S.-based non-profit agency), working in collaboration with the Royal Scientific Society of Jordan (RSS), the Biosafety and Biosecurity International Consortium (BBIC, a network of concerned individuals from 22 countries in the region), and the Moroccan Biosafety Association (MOBSA) that have developed successful sustainable projects since 2005.

Conducting the online survey related to biosafety and biosecurity issues was the most complicated endeavor in this study. Obtaining statistically significant survey data in this field, and in this region, is very difficult. The participants who did respond were relevant professionals actively involved in biorisk management activities within their respective countries; thus, the responses were considered credible and are reported herein. However, the biggest challenge, and hence a limitation of this study, was the low response rate of the online survey. Regional diversity, including differences in culture, languages, dialects, and most importantly sociopolitical unrest and perception that participation in an internationally funded survey might be harmful, resulted in a sense of fear in participants despite statements declaring that all data were confidential and anonymous.

Promoting trust between funders, regional scientists, and cooperative partners, and improving open communication about intentions and objectives for the bioengagement activity (i.e., transparency) in the region is of utmost importance. Bioengagement programs can greatly benefit by incorporating such efforts into mainstream national health and science programs, such as global aid programs focused on public health: malaria, soil and water parasitism, tuberculosis, vector-borne viruses, and HIV/AIDS. These programs over the years have gained the trust of scientific individuals and national governments; these programs can be used as a bridge in strengthening regional initiatives.

CONCLUSION

There has been a concerted effort to enhance the general awareness of biosafety and biosecurity in the life sciences in the BMENA region over the last decade. Our study findings suggest that to date, efforts have largely been focused on raising general awareness among the broad scientific community. Also, countries with actively functional Biosafety Associations and other scientifically related associations had far more such initiatives than those without such associations, but much duplication of efforts and inefficiencies of scale have been seen over the past decade. Continuing international donor interest providing opportunities for future assistance in the development of technical expertise can lead to development of local guidelines related to issues unique to the region. Given the differences across the region, local solutions are important. Country-level analyses of the local capacity-building needs are, therefore, recommended.

This study provides considerable vital information for those planning biorisk management initiatives in the region. Risk assessment and mitigation in life sciences research should be made known to a broader scientific audience, as much can be gained from similar expertise in other disciplines such as engineering, chemistry, and health physics. Efforts by donor nations and agencies to sustainably support these associations and provide biosafety trainings to their broad scientific communities may be the most efficient and effective way to build cooperative regional biosafety opportunities.

AUTHOR CONTRIBUTIONS

EK and MC conceived the idea for this project. Literature search was conducted by NA and AH, and was reviewed and analyzed by EK, JC, AE-G, KT, and MC. The survey software was developed by HG. EK analyzed survey results and overall developed the manuscript. All authors reviewed the final manuscript.

ACKNOWLEDGMENTS

We acknowledge efforts and time dedicated by country collaborators toward this project. Special thanks to the following country coordinators: Sonia Mediene from Algeria, Aly Omar from Egypt, Nisreen AL-Hmoud from Jordan, Sabah Al Momin from Kuwait, Mustapha Ennaji from Morocco, Eltayeb Ali from Sudan, Amel Benammar-Elgaaied from Tunisia, Ahmed Bashir and Mohammad Nasim Sabir from UAE, and Karim Nasher from Yemen for their personal insight to the situation and advice when required. We are also thankful to Mr. Faisal Malik and Aamir Lakhani for the secretarial assistance and coordination of activities.

FUNDING

The study was part of a larger project titled "*Scientific Engagement: Defining Gaps and Creating Opportunities for Cooperative Research and Global Security in the Broader Middle East and North Africa (BMENA) Region*" and was funded by the American Association for the Advancement of Science (AAAS) Grant # 2492 – Pat – ERC . Components of this manuscript may be found as part of the final report that was submitted to the funding agency at the end of the project. The publication of this article is to reach a wider audience and for proper recognition of the efforts by the project team.

REFERENCES

1. UNEP-GEF Project on Development of National Biosafety Frameworks. *Report of the Sub Regional Workshop for Asian Countries on: The Development of a Regulatory Regime and Administrative Systems* (2003). Available from: http://www.unep.ch/biosafety/old_site/development/devdocuments/shirazreportfinal.pdf
2. Biosafety Protocol News. *Implementation of the Cartagena Protocol on Biosafety: National Experiences and Perspectives* (2007). Available from: https://www.cbd.int/doc/newsletters/bpn/bpn-issue02.pdf
3. United Nations Environment Programme environment for development. *Development of National Biosafety Frameworks Project Algeria* (2002). Available from: http://www.unep.ch/biosafety/old_site/partcountries/DZcountrypage.htm
4. Organization for Economic Cooperation and Development. *LMOs and the Environment Proceedings of an International Conference* (2001). Available from: http://www.oecd.org/science/biotrack/31526579.pdf
5. United Nations Environment Programme environment for development. *Development of National Biosafety Frameworks Project Jordan* (2002). Available from: http://www.unep.ch/biosafety/old_site/partcountries/JOcountrypage.htm
6. Pakissan.com. *COMSTECH Hosts Inter-Ministerial Meeting on Biotechnology* (2004). Available from: http://www.pakissan.com/english/advisory/biotechnology/comstech.hosts.Interministerial.meeting.on.biotechnology.shtml
7. United Nations Environment Programme. *Islamic Republic of Iran National Biosafety Framework* (2004). Available from: http://www.unep.org/biosafety/files/IRNBFrep.pdf
8. OIE. *OIE International Conference on Bio Safety, Muscat, Oman* (2005). Available from: http://www.oie.int/doc/en_document.php?numrec=3213603
9. United Nations Environment Programme environment for development. *Development of National Biosafety Frameworks Project Yemen* (2003). Available from: http://www.unep.ch/biosafety/old_site/partcountries/YEcountrypage.htm
10. Pakistan Biological Safety Association. *The Year of Building International Biosafety Communities* (2011). Available from: http://pakbiosafety.com/The%20year%20of%20building%20international%20Biosafety%20communities%20Activity.pdf
11. Advancing Science Serving Society. *Science, Technology, and Security Policy* (2005). Available from: http://cstsp.aaas.org/InternationalMeeting/Tunisia/Tunisreportfinal.pdf
12. Pakistan Biological Safety Association. *1st Seminar on Laboratory Biosafety* (2009). Available from: http://pakbiosafety.com/activities12.htm
13. Pakistan Biological Safety Association. *2nd Seminar on Laboratory Biosafety* (2009). Available from: http://pakbiosafety.com/activities13.htm
14. Pakistan Biological Safety Association. *2nd International Biosafety Seminar* (2009). Available from: http://www.pakbiosafety.com/activities16.htm
15. Pakistan Biological Safety Association. *Certification Course Shipping Infectious Substances & Biological Specimen* (2009). Available from: http://www.pakbiosafety.com/activities5.htm
16. Pakistan Biological Safety Association. *Workshop on Laboratory Biosafety & Containment Facilities* (2009). Available from: http://pakbiosafety.com/activities4.htm
17. Pakistan Biological Safety Association. *Presentation on Fundamentals of Biosafety Cabinet* (2009). Available from: http://www.pakbiosafety.com/activities8.htm
18. Pakistan Biological Safety Association. *Biosafety Awareness Program at Indus Hospital* (2009). Available from: http://pakbiosafety.com/activities15.htm
19. Pakistan Biological Safety Association. *Participants of Biosafety Awareness Session at Indus Hospital* (2010). Available from: http://www.pakbiosafety.com/activities10.htm
20. International Biological Threat Reduction. *Laboratory Biosafety and Biosecurity Seminar Baghdad, Iraq* (2010). Available from: http://www.biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/iraq.html
21. Germfree. *2nd Annual Conference of the African Biological Safety Association Cairo* (2011). Available from: http://www.germfree.com/events/2nd-annual-conference-of-the-african-biological-safety-association
22. Pakistan Biological Safety Association. *Healthcare Waste Management Practices and Principles* (2011). Available from: http://pakbiosafety.com/activities17.html
23. Biosafety and Biosecurity International Conference. *Message from the Biosafety and Biosecurity International Conference Process* (2011). Available from: http://www.unog.ch/80256EDD006B8954/(httpAssets)/77FC749085CA1D9FC12579B4002F85D2/$file/BBICMessage.pdf
24. Middle East Scientific Institute for Security. *3rd Biosafety and Biosecurity International Conference* (2011). Available from: http://www.mesis.jo/news/159
25. World Health Organization. *Alert, Response, and Capacity Building under the International Health Regulations* (2011). Available from: http://www.who.int/ihr/ihrnews/ihrnewsissue16/en/index3.html
26. Jordon University of Science and Technology. *Biorisk Workshop* (2011). Available from: http://biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/biofitjust/bioriskwkshp.pdf
27. Recent Developments in International Laboratory Biorisk Management. *Pakistan – U.S. Bioengagement Workshop Istanbul, Turkey* (2011). Available from: http://www.biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/pakUS/recentdevintllabbrmgmt.pdf
28. Pakistan Biological Safety Association. *1st International Biosafety Conference, Building National Biosafety and Global ties* (2011). Available from: http://pakbiosafety.com/activities18.html
29. Pakistan Biological Safety Association. *Training at Sandia National Lab as Part of Biorisk Management Train the Trainer Grant, Collaborative Project of Aga Khan University, Higher Education Commission Pakistan and Sandia National Laboratory New Mexico, Albuquerque, USA* (2011). Available from: http://pakbiosafety.com/activities20.htm
30. Biosafety Clearing House. *The Libyan National Training Workshop for Effective Participation in the Biosafety Clearing-House* (2012). Available from: http://bch.cbd.int/about/news-post/?postid=104780
31. CRDF Global. *Canadian Biosafety Symposium* (2012). Available from: http://www.crdfglobal.org/news-and-events/upcoming-events/2012/05/29/2012-canadian-biosafety-symposium
32. Pakistan Biological Safety Association. *Seminar at Indus Hospital Biosafety Hospital: Status Achievable* (2012). Available from: http://pakbiosafety.com/activities22.htm
33. Dawn. *Violations of Biosafety Protocols in Labs, Health Facilities Observed* (2012). Available from: http://www.dawn.com/news/686757/violations-of-biosafety-protocols-in-labs-health-facilities-observed
34. Pakistan Biological Safety Association. *Developing Biorisk Management System in Research and Healthcare Facilities in Pakistan* (2012). Available from: http://pakbiosafety.com/activities25.htm
35. Qatar Foundation. *Unveiling the Qatar National Research Strategy* (2012). Available from: http://www.qf-arf.org/Program-PDF.php
36. Universit'a degli Studi di Milano. *Preliminary Scientific Programme 4th Annual International Symposium, Biosecurity and Biosafety: Biosecurity and Biosafety: Future Trends and Solutions* (2012). Available from: http://www.bioemergency.eu/forms/TOPICS%202012.PDF
37. Advancing Science Serving Society. *Science, Technology, and Security Policy* (2005). Available from: http://cstsp.aaas.org/files/InternationalEngagement.pdf
38. World Health Organization. *Biorisk Management Advanced Trainer Program* (2012). Available from: http://www.who.int/ihr/training/7_Session4.pdf
39. Hamad Medical Corporation's. *Biorisk Management Workshops* (2013). Available from: http://thepeninsulaqatar.com/news/latest-news/224490/hmc-conducts-two-biorisk-management-workshops
40. *Responsible Science for a Safe and Secure Society, Amman, Jordan* (2010). Available from: http://www.pakbiosafety.com/Responsible%20Science%20for%20a%20Safe%20and%20Secure%20Society%20PBSA%20webpage%20Activity%20.pdf
41. International Biological Threat Reduction. *International Perspectives on Mitigating Laboratory Biorisks Workshop Istanbul, Turkey* (2010). Available from: http://www.biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/turkey.html
42. International Biological Threat Reduction. *Introduction to Laboratory Biorisk Management Sana'a, Yemen* (2011). Available from: http://www.biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/yemenbiorisk.html

43. National Institutes of Health. *Office of Biotechnology Activities* (2011). Available from: http://oba.od.nih.gov/oba/biosecurity/meetings/March2011/Kuwait_Workshop_VTC_Summary.pdf
44. International Biological Threat Reduction. *Introduction to Biorisk Management Constantine, Algeria; Algiers, Algeria* (2011). Available from: http://www.biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/brmgmt.html
45. RSS of Jordan, EU-RL GMFF (European Commission Joint Research Centre). *1st International Workshop on Harmonization of GMO Detection and Analysis in the Middle East and North Africa (MENA) Region* (2012). Available from: http://gmo-crl.jrc.ec.europa.eu/capacitybuilding/docsworkshops/Agenda_Jordan_2012.htm
46. Biosafety, Biotechnology and GMO's Legislations in Libya. *1st International Workshop on Harmonization of GMO Detection and Analysis in MENA Region* (2012). Available from: http://gmo-crl.jrc.ec.europa.eu/capacitybuilding/docsworkshops/Jordan-2012/LIBYA.pdf
47. Ministry of Environment. *Regional Workshop on Detecting Genetically Modified Organisms in Agricultural and Food Products* (2013). Available from: http://www.moe.gov.qa/English/News/Local/Pages/Regional-Workshop-on-Detecting-Genetically-Modified-Organisms-in-Agricultural-and-Food-Products.aspx
48. Iran Technology Centre. *Workshop on Risk Assessment of GMOs* (2010). Available from: http://www.irbic.ir/index.aspx?siteid=2&pageid=160&newsview=40
49. Food and Agriculture Organization of the United Nations. *Laboratory Manual on GMO Detection* (2013). Available from: http://www.fao.org/biotech/biotech-add-edit-section/biotech-add-edit-news/biotech-news-detail/en/c/168577/
50. Jordon University of Science and Technology. *Transportation/Biorisk Workshop* (2010). Available from: http://biosecurity.sandia.gov/ibtr/subpages/pastConf/20102011/jordon/bioriskwrkshp.pdf
51. World Health Organization. *The WHO Quarterly Bulletin on IHR Implementation* (2012). Available from: http://www.who.int/ihr/ihrnews/ihrnewsissue20/en/

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 09 October 2015
doi: 10.3389/fpubh.2015.00228

Implementation and evaluation of a training program as part of the Cooperative Biological Engagement Program in Azerbaijan

April Johnson[1], Gulshan Akhundova[2], Saida Aliyeva[3] and Lisa Strelow[4]*

[1] Signature Science, LLC., Baku, Azerbaijan, [2] Technology Management Company, Baku, Azerbaijan, [3] Civilian Research Development Foundation, Baku, Azerbaijan, [4] Bechtel National Inc., Baku, Azerbaijan

A training program for animal and human health professionals has been implemented in Azerbaijan through a joint agreement between the United States Defense Threat Reduction Agency and the Government of Azerbaijan. The training program is administered as part of the Cooperative Biological Engagement Program, and targets key employees in Azerbaijan's disease surveillance system including physicians, veterinarians, epidemiologists, and laboratory personnel. Training is aimed at improving detection, diagnosis, and response to especially dangerous pathogens (EDPs), although the techniques and methodologies can be applied to other pathogens and diseases of concern. Biosafety and biosecurity training is provided to all trainees within the program. Prior to 2014, a variety of international agencies and organizations provided training, which resulted in gaps related to lack of coordination of training materials and content. In 2014 a new training program was implemented in order to address those gaps. This paper provides an overview of the Cooperative Biological Engagement Program training program in Azerbaijan, a description of how the program fits into existing national training infrastructure, and an evaluation of the new program's effectiveness to date. Long-term sustainability of the program is also discussed.

Keywords: Cooperative Biological Engagement Program, training, Azerbaijan, Ministry of Health, Ministry of Agriculture

OPEN ACCESS

Edited by:
Hillary H. Carter, Office of Cooperative Threat Reduction, USA

Reviewed by:
Li Xu, Cornell University, USA
Jennifer Gaudioso, Sandia National Laboratories, USA

****Correspondence:***
April Johnson, Cooperative Biological Engagement Program, Azerbaijan, 44 Jafar Jabbarly, Baku, AZ 1065, Azerbaijan
ajohnso5@bechtel.com

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *19 June 2015*
Accepted: *18 September 2015*
Published: *09 October 2015*

Citation:
Johnson A, Akhundova G, Aliyeva S and Strelow L (2015) Implementation and evaluation of a training program as part of the Cooperative Biological Engagement Program in Azerbaijan. Front. Public Health 3:228. doi: 10.3389/fpubh.2015.00228

Introduction and Program Background

The Cooperative Biological Engagement Program (CBEP), under the US Defense Threat Reduction Agency's broader Cooperative Threat Reduction program, works with approximately 20 countries around the world to combat biological threats [1]. Objectives of the program are to prevent the proliferation of biological weapons; consolidate and secure collections of dangerous pathogens in central live microorganism repositories; strengthen biosafety and biosecurity of laboratory facilities; and improve partner nations' ability to detect, diagnose, report, and respond to outbreaks of disease caused by especially dangerous pathogens (EDPs). EDPs are infectious agents with the potential for use as biological weapons that may result in significant harm to people or animals.

The CBEP was first started to be implemented in Azerbaijan in 2007. Bechtel National, Inc. (BNI) has been working as the integrating contractor there since 2011. The CBEP works primarily with the Ministry of Health (MoH) and State Veterinary Control Service (SVCS) within the

Ministry of Agriculture. MoH and SVCS administer the national surveillance systems for human and animal diseases, respectively, and are therefore the main recipients of program support as the implementing agencies. CBEP funded the construction or renovation of biosafety level 2 (BSL-2) laboratories throughout the country—five for human health [four regional and one Republican (national) level] and six for animal health (five regional and one Republican level). Modern diagnostic equipment was provided for these twelve laboratories (one additional laboratory for Ministry of Defense), as was training on facility and equipment maintenance, training on equipment use, and Biosafety and Biosecurity (BS&S) training. The MoH is constructing a combination BSL-2/BSL-3 Republican-level laboratory in Baku with their funds that will serve as the Central Reference Laboratory for Azerbaijan. CBEP is assisting with construction oversight and training at this facility. Construction completion is expected in 2016.

Several EDPs are endemic in Azerbaijan. There are approximately 300–500 human cases of brucellosis reported annually [2, 3]. Anthrax cases continue to be reported, in spite of livestock vaccination campaigns [4]. In a recent study, human volunteers were seropositive for tularemia [5]. One study found that not all people who are ill will seek healthcare, so reported cases of EDPs may be underestimated, as the country relies on passive surveillance from the healthcare system for disease detection [6]. Strengthening recognition of EDP cases by healthcare workers and animal health workers becomes even more important for detecting outbreaks if few people seek medical care for infectious diseases. The CBEP provides training aimed at improving rapid recognition, diagnosis, reporting, and response among key members of the Azerbaijani surveillance systems, including infectious disease specialists, human and animal clinicians, epidemiologists, and laboratory staff.

CBEP training in Azerbaijan has been delivered in two phases. From 2007 to 2010, training in clinical recognition of diseases, epidemiology, and laboratory diagnostics was provided by several agencies and organizations including the US Army Medical Research Institute of Infectious Disease, Walter Reed Army Institute of Research, US Centers for Disease Control and Prevention, UK Public Health England, and UK Animal and Plant Health Agency. The integrating contractor Black and Veatch introduced (and continues to train on) the Electronic Integrated Disease Surveillance System, which is an electronic disease reporting system integrated into the MoH and Ministry of Agriculture disease surveillance systems.

In 2011, BNI conducted a training gap analysis to establish the baseline knowledge of Government of Azerbaijan staff and the broader facility capabilities associated with CBEP in Azerbaijan. Gaps between training provided through 2010 and the competencies necessary to fulfill CBEP goals and ensure program sustainability were identified and taken into consideration when developing the new training program. Among other gaps, the analysis identified inconsistencies in materials, as well as topics that were not rigorously addressed in the original program, such as sample packaging and transport. A new training program aimed at addressing identified gaps was implemented in 2014. In the context of this new CBEP training program, BNI is delivering clinical disease recognition, epidemiology, and laboratory training in partnership with Government of Azerbaijan trainers, and continuing to provide BS&S training.

This paper describes the implementation and evaluation of the new training program developed by BNI in collaboration with the Defense Threat Reduction Agency and the Government of Azerbaijan from 2014 to 2015. Data from trainings conducted between September 2014 and April 2015 and annual BS&S trainings conducted in 2013–2014 are included in the evaluation of the program.

Methods

Implementation of Training

The CBEP training program was developed by BNI in collaboration with the governments of the US and Azerbaijan. The following disciplines, targeting both human and animal health workers, were chosen for inclusion: Clinical Recognition of Infections Caused by EDPs, Epidemiology, and three Laboratory disciplines (Bacteriology, Serology, and PCR). With the exception of Clinical trainings, Basic and Advanced level courses are being taught for each discipline, based on a needs assessment to identify appropriate trainees for the advanced courses. Since both ministries agreed to a One Health approach to Epidemiology training, the trainee pool for this discipline comprises an equal number of participants from the MoH and the SVCS. The Epidemiology courses include a mixture of joint lectures and exercises, as well as separate Ministry-specific breakout sessions. BNI has provided annual biosafety and biosecurity (BS&S) training for laboratory staff since 2013, and will deliver Biosafety Officer training to designated trainees. Together with Black & Veatch, BNI will also provide BS&S training to Central Reference Laboratory staff.

To ensure that all Azerbaijani training needs were taken into account in the CBEP training program, training sub-working groups (TSWGs) were established with both Ministries. These groups met twice monthly between April and September 2014. The TSWGs consisted of four individuals from SVCS and nine individuals from MoH. The working groups identified subject matter experts for each discipline (Clinical, Epidemiology, Bacteriology, PCR, Serology, and BS&S) to assist with material development and review. Using pre-qualification criteria that were developed for each subject discipline, the working groups also identified the trainees who would most benefit from the training and represent the broader target audience geographically.

Training Materials

Training materials are based on modules previously developed by CBEP subject matter experts and provided to BNI as Government-furnished information adapted for use in Azerbaijan. BNI also developed training materials when previously developed materials were not available. Subject matter experts identified by the TSWGs reviewed the training

materials, adapting them and providing examples specific to Azerbaijan as needed. All materials were approved by the Defense Threat Reduction Agency, MoH, and SVCS before training implementation. Efforts were made to use the most up-to-date information available in the English language scientific literature, and to reference international standards where applicable. Trainees are encouraged to report inaccurate translations or other errors noted during training on comment forms, so as to improve materials for future delivery. Relevant consensus changes suggested by trainees and trainers will continue to be incorporated into the materials throughout delivery of the courses.

All trainees are provided with a hard copy of the training materials to use during the training and will receive electronic versions of the materials once all training is delivered and the materials are finalized (i.e., no further translation changes are made during course delivery). At the request of MoH, training materials (slides and slide notes, plus additional background materials) will also be compiled into bound booklets to serve as a reference for future trainers.

Trainers

Prior to training implementation in September 2014, the MoH and SVCS identified co-trainers to administer training alongside BNI. The training program will transition entirely to the Government of Azerbaijan in January 2016, so it is important to help develop the Government of Azerbaijan trainers quickly. All Government of Azerbaijan co-trainers participated in an Adult Learning Principles course conducted by BNI to improve their training skills and to ensure a consistent approach to training delivery among all trainers. After observing one course delivered exclusively by a BNI expatriate trainer, these Government of Azerbaijan trainers co-deliver an increasing proportion of course content with each subsequent training event until they are training 100% of the course. This occurs during the last scheduled course, if not sooner. Individual co-trainers will have covered every topic in the discipline by the end of the scheduled trainings, and will continue to deliver this training beyond CBEP's period of engagement, ultimately sustaining the training program in Azerbaijan for years to come.

Training events are delivered by a team comprising one BNI expatriate trainer, 1–3 local national BNI training facilitators, and 1–4 Government of Azerbaijan co-trainers. The total number of trainers for any given event depends on the course discipline and number of trainees. Prior to each event, the training teams meet to allocate assignments, review course material as needed, and to clarify any logistical issues. During the training, teams meet as necessary to address any training or logistical issues and to discuss how the training delivery and training materials can be improved.

Training conducted by expatriate trainers requires interpretation from English; training conducted by local national facilitators and Government of Azerbaijan trainers is conducted in Azerbaijani. The MoH-identified co-trainers are authorized by the Azerbaijani State Doctors Improvement Institute (SDII) to provide continuing education course credit to doctoral level staff who successfully pass CBEP post-training tests. MoH co-trainers who are registered with the Baku Base Medical College #2 (BBMC2) are likewise authorized to provide continuing education course credit to laboratory technician level staff who successfully pass CBEP post-training tests.

Trainee Identification

Trainees were selected by the TSWGs at the start of the program, with changes made as necessary based on trainee availability. To cover the widest geographic area in a limited amount of time, trainees from each rayon (region) were chosen for each discipline (clinical human, clinical vet, human epidemiology, and veterinary epidemiology) based on their job duties; more than one individual was targeted in each rayon to provide redundancy in the event that a trainee leaves the system. Four individuals per laboratory were targeted for Basic training in each discipline; from this pool, two trainees will be selected for advanced training based on individual job duties and performance during the Basic level course. Trainees are expected to train eligible participants in their rayons or laboratories who did not participate in CBEP training events.

Pre-qualification criteria were developed to identify the most appropriate trainees. For example, Epidemiology trainees must be (1) employed by the relevant ministry, (2) responsible for conducting epidemiological investigations of EDP cases, (3) responsible for data entry into the Electronic Integrated Disease Surveillance System, (4) responsible for advising on and implementing prevention and control measures against EDPs for the rayon, (5) 60 years of age or less, and (6) willing to train others. Similarly, Clinical trainees must be (1) employed by the relevant ministry, (2) responsible for diagnosing cases of disease suspected to be caused by EDPs, (3) 60 years of age or less, and (4) willing to train others.

Training Delivery

A comprehensive training schedule covering 15 months (September 2014–November 2015) was developed by BNI in collaboration with the MoH and SVCS. Training events for the laboratory disciplines are held at the 12 CBEP-engaged laboratories. This includes five regional veterinary laboratories (Zonal Veterinary Laboratories), four regional human health laboratories (Anti-Plague Division Laboratories), and three laboratories in Baku (the Republican Anti-Plague Station, Republican Veterinary Laboratory, and the Ministry of Defense Center for Sanitary-Epidemiological Control Laboratory). Clinical and Epidemiology trainings are held in conference rooms in hotels in five cities throughout Azerbaijan (Baku, Ganja, Guba, Gakh, and Lankaran). Trainees who live more than 1 h from the site are provided with lodging at the hotel where the training will take place. Laboratory course size is limited to approximately four trainees for Basic and two trainees for Advanced courses. Clinical courses are limited to approximately 20 trainees. Epidemiology trainings are conducted for approximately 16 trainees at a time, half of whom are human health epidemiologists and half of whom are veterinary epidemiologists. Annual BS&S training takes place at each of the 12 laboratories for an average of 10 trainees per laboratory.

Evaluation of Training

The most widely used method for evaluating training programs is "The Four Levels of Learning Evaluation" developed by Kirkpatrick in 1959 and later revised [7]. The four levels of evaluation are *Reaction, Learning, Behavior*, and *Results*. *Reaction* was assessed by administration of anonymous trainee evaluation forms that were filled out daily during training events. *Learning* was assessed by way of pre- and post-training test scores. *Behavior* and *Results* require long-term evaluation and are further addressed in the Discussion Section.

Complete data is available for courses administered by BNI between September 2014 and April 2015 for the Clinical, Epidemiology, and Laboratory disciplines, as well as annual BS&S training events conducted in 2013 and 2014.

Reaction

The first level of learning represents the satisfaction of the trainees with the training. This was evaluated by means of anonymous trainee evaluation forms. Standardized questionnaires were developed and are administered to trainees at the end of each training day to solicit their opinions on the content of the training, quality of the training, and knowledge and skill levels of the trainers on a five-point scale (*Poor*/*Below Average*/*Met Expectations*/*Above Average*/*Excellent*). Three-point scales (*Enough*/*Just Right*/*Not Enough*, *Basic*/*Appropriate*/*Too Advanced,* and *Yes*/*Not Sure*/*No*) are used to determine if allotted time, content, and practical exercises were sufficient; if the material was too basic or advanced; and if the material is relevant to their current positions. Open-ended questions provide an opportunity for trainees to comment on the lectures they liked most or least, and to seek input on how to improve the training. Input was sought daily while the information was fresh, rather than waiting until the end of the event, when trainees would be less likely to recall earlier trainers and lectures. Input from each day was summarized and compiled for a complete evaluation of each event. The data presented herein is derived from overall summaries of trainee evaluations from Clinical, Epidemiology, and Laboratory trainings conducted between September 2014 and April 2015.

Learning

Pre- and post-training tests were used to evaluate the knowledge gained during a given course. A pre-training test was administered on the first day of the course to gauge baseline knowledge, and the same test was administered at the end of the course to evaluate knowledge gain. Tests are usually 25 questions in length (range 15–30 questions) and are comprised of multiple choice and some true/false questions. Data from trainees who did not complete both the pre- and post-training tests are not included in the summary.

Results

Implementation of Training

Planning for the BNI training program began in April 2014. A total of 74 training events (targeting 584 total trainees) were planned from September 2014 to December 2015 in the Clinical Recognition of Infections caused by EDPs, Epidemiology, Bacteriology, Serology, and PCR disciplines. EDPs covered in the training program are listed in **Table 1**. Between September 2014 and April 2015, 46 training events were conducted for 386 trainees. From 2013 to 2014, 24 BS&S annual refresher training events were held for a total of 196 participants.

TABLE 1 | Especially Dangerous Pathogens (EDPs) covered in the CBEP training program.

Human health	Animal health	Zoonoses
Yersinia pestis (plague)	Capripox virus	Avian influenza virus
Francisella tularensis (tularemia)	Newcastle disease virus	*Brucella* species (brucellosis)
Crimean-Congo hemorrhagic fever virus	African swine fever virus	*Bacillus anthracis* (anthrax)
Tick-borne encephalitis virus	Classical swine fever virus	*Coxiella burnetii* (Q fever)
Smallpox virus	Foot and mouth disease virus	
Clostridium botulinum	*Burkholderia mallei* (glanders)	
	Rinderpest virus	
	Peste des Petits Ruminants virus	

TABLE 2 | Total number of MoH and SVCS co-trainers active in the program as of April 2015.

Categories	No. of MoH trainers	No. of SVCS trainers
Clinical recognition	2	2
Epidemiology	3	4
Bacteriology	3	3
Serology	2	3
PCR	3	3
Biosafety and biosecurity	2	2
Total	15	17

The list of co-trainers identified by the Government of Azerbaijan evolved over the course of the program, as competing responsibilities arose or trainers left their job posts. As new trainers were identified, additional courses in Adult Learning Principles were provided to ensure that all trainers were equipped to provide training, and all materials were provided well in advance of training to allow trainers to gain familiarity with the materials. A total of six events in Adult Learning Principles were provided to small groups of Government of Azerbaijan co-trainers. Refer to **Table 2** for the numbers of Government of Azerbaijan trainers in the program.

Evaluation of Training

Reaction

Since evaluation forms were requested to be filled out daily, response rates tended to be fairly high. However, trainees did not always answer every question, as noted by the variable numbers of responses in **Figures 1–6**.

Overall, approximately 97% of trainees felt that the training covered topics that would be useful for them in their daily work, and 78% felt that the level of the material was appropriate (neither too advanced nor too basic). About 80% felt there was

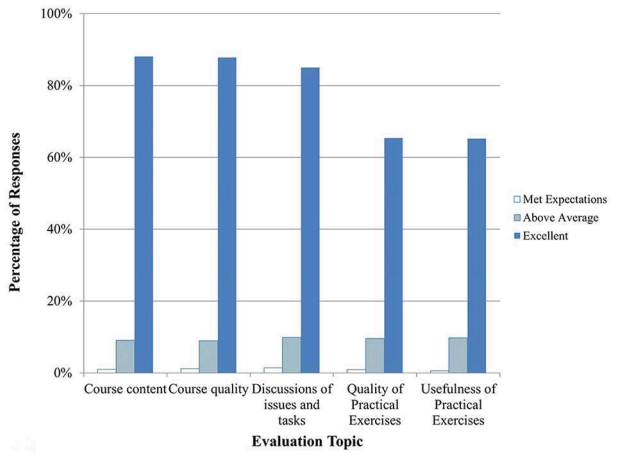

FIGURE 1 | Clinical training evaluation by trainees (n = 873–926 total daily responses for each question). Note that no responses were marked as poor or below average so those categories are not shown.

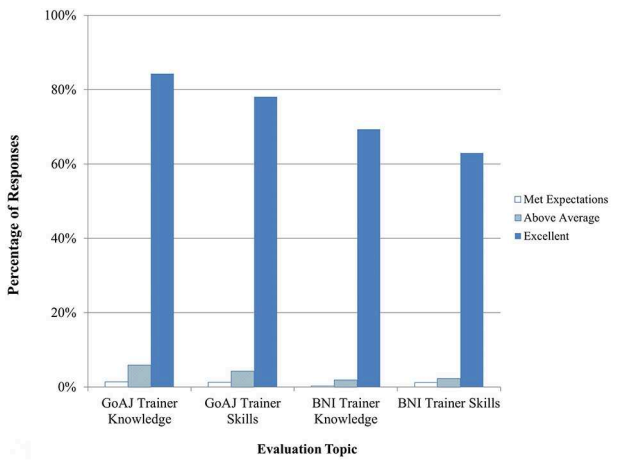

FIGURE 2 | Trainee feedback on Clinical trainer knowledge and skills (n = 909–1687 total daily responses for each question). The number of N/A responses to these questions is not shown.

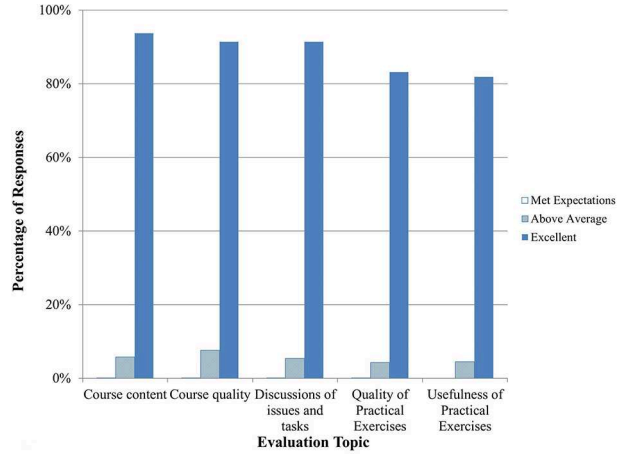

FIGURE 3 | Epidemiology training evaluation by trainees (n = 1018–1107 total daily responses for each question). Note that no responses were marked as poor or below average so those categories are not shown.

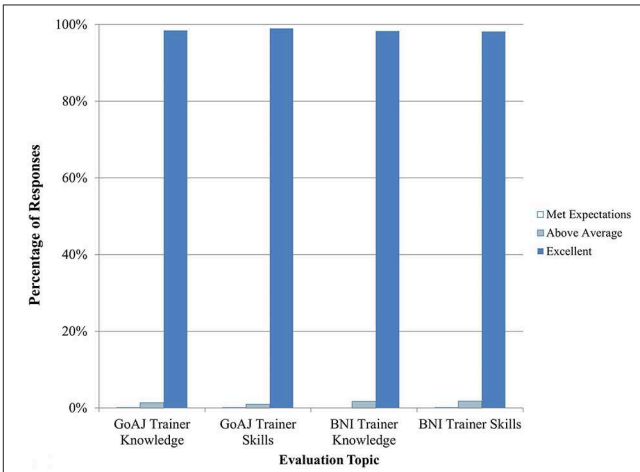

FIGURE 4 | Trainee feedback on Epidemiology trainer knowledge and skills (n = 1810–2011 total daily responses for each question). The number of N/A responses to these questions is not shown.

sufficient time to cover the material being presented, and that the amount of content and practical exercises was sufficient. Results of questions on course content and quality and trainee perceptions of the knowledge and skill levels of both BNI and Government of Azerbaijan trainers can be found in **Figures 1**–**6**.

Clinical training

Between September 2014 and April 2015, 13 courses in Clinical Recognition of Infections Caused by EDPs were delivered, seven for physicians and six for veterinarians. A summary of trainee feedback is shown in **Figures 1**, **2**. Over 80% of trainees responded that the course content, quality, and discussions were excellent. 65% of trainees felt that the practical exercises were excellent in quality and usefulness, while about 24% responded Not Applicable (N/A). Hands-on practical exercises for Clinical courses are challenging to implement since human patients cannot be used and animals cannot be brought to training sites; therefore, practical exercises for this discipline are limited to case studies and scenario exercises.

Epidemiology

Seven courses in Epidemiology were conducted between September 2014 and April 2015 for mixed audiences of human and veterinary epidemiologists. Practical exercises in this course included case studies and exercises. Overall, trainees were satisfied with the course content and quality (**Figure 3**). They enjoyed the joint One Health approach and asked for additional lectures to be provided as joint trainings. Trainees were very satisfied with the knowledge and skills of both the BNI and Government of Azerbaijan trainers (**Figure 4**).

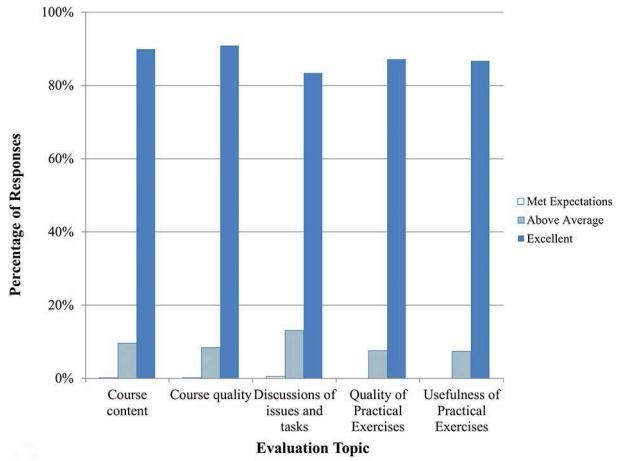

FIGURE 5 | Laboratory training evaluation by trainees (n = 421–427 total daily responses for each question). Note that no responses were marked as poor or below average so those categories are not shown.

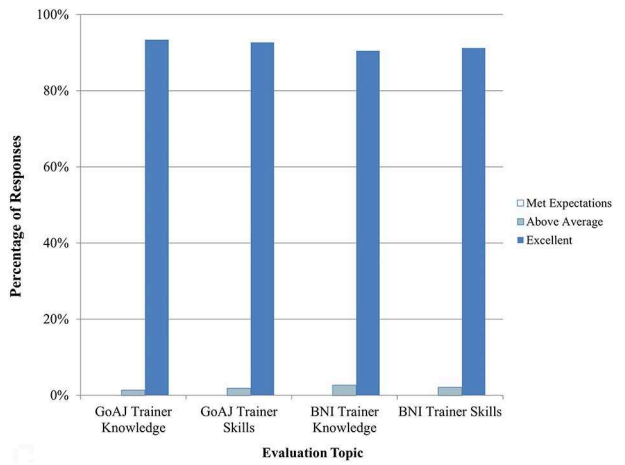

FIGURE 6 | Trainee feedback on Laboratory trainer knowledge and skills (n = 262–485 total daily responses for each question). The number of N/A responses to these questions is not shown.

Laboratory training

Nineteen Laboratory training events were conducted between September 2014 and April 2015. Practical exercises were conducted to reinforce skills in pipetting, setting up and running assays, and interpreting results. About 87% of trainees rated the exercises as excellent in terms of quality and usefulness and 90% or more felt that the course content and quality and trainer knowledge and skills were excellent (**Figures 5**, **6**).

Learning

Of 386 trainees, pre- and post-training test scores were available for 375 trainees (97%). Overall, post-training test scores were higher than pre-training scores (**Table 3**), indicating an increase in trainee knowledge. Due to the small class sizes, data from the MoH and SVCS trainees were combined for the Laboratory disciplines.

Annual biosafety and biosecurity refresher training

From 2013 to 2014, 196 total trainees from the twelve CBEP-engaged laboratories participated in annual BS&S refresher training (97 in 2013 and 99 in 2014). The median scores and interquartile ranges from pre- and post-training tests are shown in **Figure 7**. The median pre-training test score in 2013 was 30% and the median post-training test score was 75%. In 2014, the test scores increased from a median of 57% on the pre-training test to 82% on the post-training test.

Discussion

Since the beginning of CBEP engagement in Azerbaijan, 1710 individual human and animal healthcare workers and laboratory staff have received training. Since implementation of the new training program in September 2014, the program has trained 32 Government of Azerbaijan trainers, including at least two trainers for each discipline from each ministry. Between September 2014 and April 2015, 386 MoH, and SVCS staff members were trained in Clinical Disease Recognition, Epidemiology, and Laboratory disciplines. An additional 400 trainees are expected to have received training by the end of 2015.

Evaluation of the Training Program

Trainees rated the training program content and quality highly on anonymous feedback forms and the majority felt the information trained would be useful in their work. Their responses to open-ended questions showed which topics were of most interest and use to the trainees. Many respondents asked for additional trainings in the future.

Overall, median scores for pre-training tests increased for each discipline. Median Epidemiology test scores are uniformly lower than those in other disciplines, and especially for veterinary staff; the median Epidemiology post-training test scores fell below CBEP's threshold for a passing grade (70%). During the training events, it became evident that the epidemiology content was new to most of the trainees and that the concepts discussed were not routinely used in daily activities. As a result, further discussions were held with both ministries to identify the most effective approach to follow-on training.

Annual BS&S training pre-training test scores increased from 30% in 2013 to 57% in 2014. Fifty-three percent of those trained in 2014 were also trained in 2013. This increase in scores from 2013 to 2014 suggests that there might be some residual knowledge from the previous years' training or shared knowledge by trained staff to new staff; data from annual BS&S training events in 2015 will be analyzed to see if pre-training test scores continue to rise within this trainee pool.

Training programs should result in behavior change and positive outcomes, which are the third and fourth levels of Kirkpatrick's training evaluation model and are more difficult to assess than the first and second levels [7]. There are six local national BNI staff members who visit the twelve CBEP-engaged laboratories twice a month to observe regular laboratory practices and behaviors, to provide mentorship as necessary, and to administer proficiency testing in Laboratory disciplines. A laboratory assessment tool was recently implemented to

TABLE 3 | Median pre- and post-test scores for training events between September 2014 and April 2015, with 25–75% interquartile ranges.

Category	No. of courses	No. of trainees	No. Trainees with pre/post-test scores	Pre test % (Q1–Q3)	Post test % (Q1–3)	% increase (Q1–Q3)
Clinical recognition of infections caused by EDPs (MoH)	7	106	100	47.5 (36 – 53)	77 (67 – 86)	30 (21 – 40)
Clinical recognition of infections caused by EDPs (SVCS)	6	97	97	47 (36 – 59)	75 (64 – 82)	25 (13 – 39)
Epidemiology (MoH)*	7	60	59	45.5 (36.5–53)	69 (59 – 78)	23 (15.5–28.5)
Epidemiology (SVCS)*	7	53	53	34.5 (30.5–41.5)	54.5 (47 – 67)	19.5 (9 – 29)
Basic PCR	5	19	17	32 (16 – 48)	80 (80 – 88)	40 (20 – 60)
Advanced PCR	4	10	8	40 (32 – 50)	73.5 (70 – 83)	28.5 (24–45.5)
Basic serology	4	20	20	27 (18–28.5)	87 (80 – 93)	66 (47 – 71)
Advanced serology	2	4	4	44.5 (41.5–48)	76 (61–88.5)	30.5 (13 – 46)
Basic bacteriology	2	10	10	50 (36 – 59)	70 (61–77.5)	17.5 (10 – 25)
Advanced bacteriology	2	7	7	55 (42.5–57.5)	85 (82.5–87.5)	30 (27.5–42.5)

**Tests were administered each week of a 2-week course; scores represent an average of the two pre-training test and the two post-training test scores.*

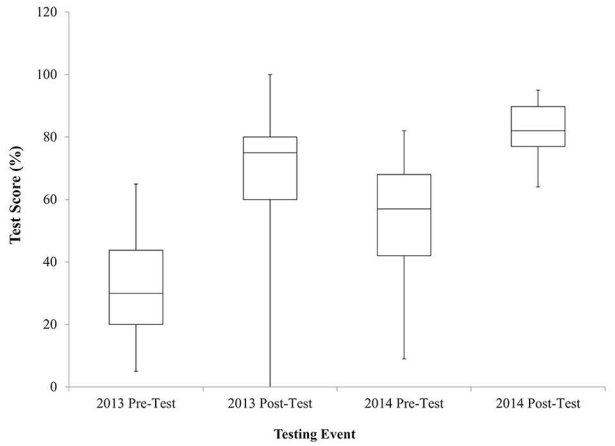

FIGURE 7 | Quartile values of median pre- and post-training test scores for Annual BS&S Refresher training events for 2013 and 2014. Note that one individual in 2013 scored a 0 on the post-training test which was later determined to be due to an inability to read Azerbaijani. Following this result, a Russian translation was also made available, or the tests were read aloud to individuals who could not read Azerbaijani. Excluding this individual, the lowest score on a post-training test was 30%.

quantitatively assess performance over time, but only at the laboratory level and not at the individual trainee level. The PCR proficiency testing program was re-implemented by BNI in October 2014; the proficiency testing programs in Bacteriology and Serology are scheduled to begin in June 2015. The proficiency testing program allows ongoing assessments of individual trainee performance and behavior in the areas of BS&S and laboratory diagnostic testing. Assessing behavior change among Clinical and Epidemiology trainees is more challenging, as BNI interacts less frequently with those individuals and they are more geographically dispersed. BNI plans to administer post-training questionnaires and tests to evaluate learning retention, and to assess attitudes and practices in these trainee pools.

One goal of the CBEP training program is a surveillance system that can rapidly and effectively recognize, report, diagnose, and respond to EDPs; adherence to appropriate BS&S practices is another critical result. EDP outbreaks occur only sporadically—it is therefore problematic to measure success of such outcomes in a natural setting. However, several cases of EDPs have been diagnosed by Government of Azerbaijan personnel at the CBEP-engaged laboratories using techniques introduced by CBEP. In addition, the Defense Threat Reduction Agency assessed a vertical slice of the human and animal disease surveillance systems by means of an operational demonstration in March 2014. In this exercise, a hypothetical anthrax outbreak scenario was presented to veterinary and human clinicians and epidemiologists to assess how they would respond. The assessment included their ability to gather appropriate physical exam histories; create a differential diagnosis; collect appropriate diagnostic samples in a safe manner; package and transport the samples; and to correctly report the disease in the Electronic Integrated Disease Surveillance System. To demonstrate diagnostic and biosafety practices, laboratory staff were assessed on how they tested non-pathogenic samples of the specific disease agent using CBEP-trained techniques. Overall, although both the human and animal health systems were found to be acceptable, areas for additional training were identified and incorporated into the current training program or are being addressed via mentorship visits.

Training Challenges

There are several obstacles to the long-term success of the CBEP training program in Azerbaijan including an aging workforce, a limited pool of qualified trainers, a lack of a post-graduate training entity for SVCS staff, and limited resources designated for EDP training.

As salaries are low in comparison with non-state sectors and recruitment/retention of newly graduated or young staff is consequently difficult, resulting in a workforce with many staff members close to retirement age. An initial cutoff of 50 years

of age was proposed for trainees in Clinical Recognition and Epidemiology disciplines, but neither ministry felt that they could identify sufficient numbers of trainees under 50 years of age. The gap analysis conducted in 2011 found that 42% of 110 human and veterinary facility staff who volunteered their age was over the age of 50. As existing personnel retire and new personnel are hired, ongoing training will be necessary to bring new staff up to speed.

The turnover of Government of Azerbaijan trainers in the training program observed to date presents an ongoing risk to training continuity if the trend continues. There is a small pool of qualified trainers, many of whom have competing responsibilities as part of their routine work duties. Of 27 trainers initially identified by MoH in fall of 2014, only 13 are still trainers; two new trainers have also been added. The SVCS has likewise made several changes to the trainer list: of 20 originally trainers, five were removed and three were added, one of whom was subsequently removed. Such ongoing changes reduce the amount of time available for newly identified trainers to gain familiarity with the materials and build experience in delivering training before the transition of the CBEP training program to the Government of Azerbaijan.

At present, the SVCS has neither designated an institute to provide continuing education for veterinarians, nor mandated a certain amount of regular continuing education. SVCS epidemiologists and veterinarians are only trained during veterinary school. Veterinary epidemiologists receive most of their training on-the-job, and neither group receives any formal continued education (unlike the MoH doctors, who must receive training every five years at the SDII). This lack of a formal post-graduate training program means that veterinarians are not receiving refresher training or keeping up with new advancements in the field. Additional efforts will be needed to ensure that the CBEP-provided EDP training courses continue.

Resources for training are limited, and therefore additional funding must be requested for ongoing training to be sustained by each Ministry starting in 2016. BNI is working with the ministries to provide budget estimates for the training program in order to ensure that sufficient funds are requested.

Future CBEP Training

The MoH has identified two training entities to continue the CBEP trainings. The SDII provides ongoing continuing education to physicians, epidemiologists, and laboratory doctor level staff, and serves as the licensing agency for human healthcare workers. Doctors receive training and take the licensing examination every 5 years. As of September 2014, all MoH trainees in CBEP disciplines (see **Table 3**) who receive a post-training test score of 50% or more receive SDII credit. MoH plans to continue training CBEP-provided courses through SDII with the use of MoH trainers starting in September 2015. Similarly, the BBMC2 serves as the post-graduate training entity for MoH laboratory technicians. One MoH trainer in each discipline is registered with BBMC2, which allows them to provide course credit to laboratory technician trainees. These trainers will continue to provide training to these individuals at the Anti-Plague Division regional laboratories since the BBMC2 does not have the same diagnostic equipment as the CBEP-engaged laboratories. Training at BBMC2 is traditionally theoretical and not practical, so training at the laboratories will allow technicians to continue to receive practical hands-on training in EDP diagnostic testing.

As previously discussed, the SVCS does not have a post-graduate training institute, although the issue has been raised with the Minister of Agriculture. In the meantime, the SVCS plans to request funding to start conducting training using the CBEP-trained trainers and possibly top-performing trainees from the training events conducted during the current CBEP training program. Without a designated training institute, it will be more challenging to continue the training program after transition of the training program to SVCS.

All relevant CBEP training materials and records will be provided to each ministry. Training materials will be finalized in conjunction with Government of Azerbaijan trainers to ensure that materials are comprehensive and sufficiently detailed that newly identified trainers can easily pick up the materials to conduct training. Azerbaijan-specific and regional examples are being included to ensure that the materials are relevant.

An electronic training database will also be shared with each ministry. The database outlines all the trainings conducted to date, the trainees who participated, and pre- and post-training test scores. This will not only pertain to the recent CBEP training program, but to all trainings conducted to date by CBEP collaborators and integrating contractors. Training records will help the Government of Azerbaijan to identify participants for future training events and to record progress.

Conclusion

Overall the CBEP training program has been successful, as evidenced by positive trainee feedback, Government of Azerbaijan's desire to continue training CBEP courses in the future, and MoH's willingness to integrate CBEP training into current national training curricula. Improvements in pre- and post-training test scores also indicate that training has been effective. Ongoing evaluation will help to identify changes in behavior and in the ability of the human and animal disease detection and response systems to recognize and respond to EDP outbreaks. Continued training will ensure that newly hired personnel are integrated into the surveillance system, that Government of Azerbaijan trainers maintain their training skills, and that those engaged in surveillance activities maintain their ability to respond to EDPs in the future.

Acknowledgments

This work was funded by the Defense Threat Reduction Agency through the Collaborative Biological Engagement Program. The authors express their gratitude to Dr. Viktor Gasimov (Ministry of Health) and Dr. Siala Rustamova (State Veterinary Control Service under the Ministry of Agriculture) for program support. The authors are grateful to the AJ scientists engaged in the CBEP research program for their productive work. The authors would also like to acknowledge Sonya Narodny for assistance with review and formatting of the manuscript.

References

1. Young S, Willis H, Moore M, Engstrom J. *Measuring Cooperative Biological Engagement Program (CBEP) Performance; Capacities, Capabilities and Sustainability Enablers for Biorisk Management and Biosurveillance*. (2014). Available online at: http://www.rand.org/content/dam/rand/pubs/research_reports/RR600/RR660/RAND_RR660.pdf (Accessed May 12, 2015).
2. Abdullayev R, Kracalik I, Ismayilova R, Ustun N, Talibzade A, Blackburn J. Analyzing the spatial and temporal distribution of human brucellosis in Azerbaijan (1995 – 2009) using spatial and spatio-temporal statistics. *BMC Infect Dis.* (2012) **12**:185. doi: 10.1186/1471-2334-12-185
3. Ismayilova R, Nasirova E, Hanou C, Rivard R, Bautista C. Patterns of Brucellosis infection symptoms in Azerbaijan: a latent class cluster analysis. *J Trop Med.* (2014) **2014**:1–4. doi: 10.1155/2014/593873
4. Kracalik I, Abdullayev R, Asadov K, Ismayilova R, Baghirova M, Ustun N, et al. Changing patterns of human anthrax in Azerbaijan during the post-Soviet and preemptive livestock vaccination eras. *PLoS Negl Trop Dis.* (2014) **8**:e2985. doi:10.1371/journal.pntd.0002985
5. Clark D, Ismailov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, et al. Seroprevalence of tularemia in rural Azerbaijan. *Vector Borne Zoonotic Dis.* (2012) **12**:558–63. doi: 10.1089/vbz.2010.0081
6. Clark D, Ismayilov A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, et al. Under-utilization of health care services for infectious diseases syndromes in rural Azerbaijan: a cross-sectional study. *BMC Health Serv Res.* (2011) **11**:32. doi: 10.1186/1472-6963-11-32
7. Kirkpatrick D, Kirkpatrick J. *Evaluating Training Programs: The Four Levels.* San Francisco, CA: Berrett-Koehler Publishers (2006).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

PERSPECTIVE
published: 26 February 2016
doi: 10.3389/fpubh.2016.00025

Strengthening Biosecurity in Iraq: Development of a National Biorisk Management System

*Mahdi F. H. Al Jewari[1] and Gregory D. Koblentz[2]**

[1] Iraqi National Monitoring Authority, Ministry of Science and Technology, Baghdad, Iraq, [2] School of Policy, Government, and International Affairs, George Mason University, Fairfax, VA, USA

Since 2004, the Republic of Iraq has undertaken a concerted effort to comply with all of its international obligations to prevent the proliferation and the use of chemical, biological, radiological, and nuclear (CBRN) weapons. A centerpiece of this effort is Iraq's development of a National Biorisk Management System. The Iraqi National Monitoring Authority (INMA), which is responsible for CBRN security and non-proliferation in Iraq, has played a key role in establishing this system. This article provides an overview of Iraq's international non-proliferation commitments, describes the legal and organizational steps it has taken to implement these commitments, and examines current initiatives to strengthen Iraq's biosecurity.

OPEN ACCESS

Edited by:
Jeanne Marie Fair, Defense Threat Reduction Agency, USA

Reviewed by:
Paras Jain, Albert Einstein College of Medicine, USA
Joe Posid, Centers for Disease Control and Prevention, USA

***Correspondence:**
Gregory D. Koblentz
gkoblent@gmu.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *14 August 2015*
Accepted: *08 February 2016*
Published: *26 February 2016*

Citation:
Al Jewari MFH and Koblentz GD (2016) Strengthening Biosecurity in Iraq: Development of a National Biorisk Management System. Front. Public Health 4:25. doi: 10.3389/fpubh.2016.00025

Keywords: Iraq, biosecurity, public health, bioengagement, biodefense, global health, biosafety, biorisk

IRAQ'S IMPLEMENTATION OF ITS NON-PROLIFERATION COMMITMENTS

Since 2004, Iraq has strengthened its commitment to the non-proliferation of CBRN weapons. Iraq is now party to all of the major international non-proliferation treaties (see **Table 1**). Iraq welcomed the adoption of United Nations Security Council Resolution (UNSCR) 1540 and submitted its first National Report to the 1540 Committee on April 13, 2005. In 2014, Iraq submitted a report to the committee on its experiences, best practices, and lessons learned from implementation of UNSCR 1540 (1).

Iraq has taken a series of practical steps to implement its obligations under international non-proliferation treaties to prevent the proliferation of weapons of mass destruction and their means of delivery to states and non-state actors. Article 9, paragraph e of Iraq's constitution, adopted by popular referendum in 2005, states that: "the Iraqi government shall respect and implement Iraq's international obligations regarding the non-proliferation, non-development, non-production and non-use of nuclear, chemical and biological weapons and shall prohibit associated equipment, material, technologies and delivery systems for use in the development, manufacture, production, and use of such weapons."

In 2006, Iraq began developing national legislation to implement and enforce its treaty and constitutional commitments to prevent the proliferation of CBRN weapons and their means of delivery. The draft law was reviewed by the International Atomic Energy Agency (IAEA) and the Organization for the Prohibition of Chemical Weapons (OPCW), the international organization in charge of implementing the Chemical Weapons Convention (CWC). The Iraqi parliament passed the National Monitoring Authority for Non-Proliferation Act No. 48 (2012) on February 16, 2012 and the legislation went into effect on September 1, 2012. The Act prohibits the development, production, possession,

TABLE 1 | Iraq's non-proliferation commitments.

Treaty	Date of signature	Date of ratification or accession
Geneva Protocol		September 8, 1931
Partial Test Ban Treaty	August 13, 1963	December 3, 1964
Nuclear Non-Proliferation Treaty (NPT)	July 1, 1968	October 29, 1969
Biological Weapons Convention (BWC)	May 11, 1972	June 19, 1991
Comprehensive Nuclear-Test-Ban Treaty (CTBT)	August 19, 2008	September 26, 2013
IAEA's Additional Protocol	October 9, 2008	October 10, 2012
Chemical Weapons Convention (CWC)		January 13, 2009
Hague Code of Conduct (HCOC)	June, 2011	June, 2011
Convention on the Physical Protection of Nuclear Material		July 7, 2014

transfer, or use of CBRN weapons and their means of delivery and establishes a system to control the import, export, and transfer of dual-use materials. Violations of the law can be punished by fines of up to 200 million Iraqi dinars and life imprisonment.

IRAQI NATIONAL MONITORING AUTHORITY

The National Monitoring Authority for Non-Proliferation Act No. 48 (2012) established the INMA, part of the Ministry of Science and Technology, as the main agency charged with implementing the new legislation. INMA was formed on the basis of the National Monitoring Directorate that had been created in 1993 to serve as Iraq's official liaison with the IAEA and the United Nations Special Commission (UNSCOM), the organization charged by the United Nations Security Council after the 1991 Persian Gulf War with overseeing Iraq's disarmament in the fields of missiles and chemical and biological weapons. INMA's overarching goal is to establish and maintain a national system of monitoring, investigation, and inspection that enables Iraq to comply with its national and international non-proliferation obligations. INMA is organized into five main functional departments (Nuclear, Biological, Chemical, Means of Delivery, and Import/Export) and a number of supporting units (Operations, Research and Studies, Administrative, Legal and Financial, Public and International Relations, and Chairman's Office).

Iraqi National Monitoring Authority has three primary functions: compliance, monitoring of dual-use materials, and capacity building. First, INMA is the lead agency for interacting with international non-proliferation organizations and is responsible for ensuring Iraqi compliance with its international non-proliferation commitments. To achieve this objective, INMA is the designated point of contact for the OPCW, the BWC's Implementation Support Unit (ISU), the IAEA, the 1540 Committee, and the Comprehensive Test Ban Treaty Organization. INMA is also responsible for preparing declarations and reports required by these organizations and accompanies inspection teams from these organizations during their visits to Iraq. In the context of the BWC, INMA compiles and submits annual confidence-building measure reports to the ISU and participates in meetings of experts, meetings of state parties, and review conferences (2).

Iraqi National Monitoring Authority's second major function is to develop regulations and mechanisms to monitor the production, use, storage, import, export, and shipping of dual-use materials to ensure their safe, secure, and peaceful application in accordance with national and international laws and standards. INMA, in cooperation with other Iraqi ministries, has developed a national system to control the import, export, and movement of dual-use materials. Iraq's list of controlled dual-use materials is based on lists maintained by the Missile Technology Control Regime, Nuclear Suppliers Group, Australian Group, Wassenaar Arrangement, and EU Law No. 2000/1334. All Government institutions and private sector companies and individuals who intend to import or export dual-use items must obtain a license from the Ministry of Trade (MOT). MOT is responsible for checking the accuracy of the information and forwarding the information to INMA for registration. If INMA grants permission for the requested license, MOT sends a copy of the license to the General Authority for Customs (GAC). GAC matches the shipping manifests of dual-use materials being transferred with the information in the license and informs its inspectors at border crossings who are responsible for checking the imported or exported materials against the license. If the license is for an import, INMA conducts a follow-up inspection with the end user of the material and makes any declaration required under international non-proliferation treaties. The Ministry of Transportation issues instructions regarding the transfer of chemical, biological, radioactive, and hazardous materials, ensures carriers comply with safety and security standards, and reports any transfers or retransfers of dual-use materials to INMA.

Iraqi National Monitoring Authority's third major task is to develop and implement capacity building programs to enhance Iraq's ability to prevent the proliferation of CBRN weapons to states and non-state actors and strengthen Iraq's preparedness for responding to the use of such weapons. To fulfill this function, INMA has started to hold workshops for managers and staff of different Iraqi ministries to raise awareness of the threat of CBRN weapons, the identification of dual-use materials, and implementation of UNSCR 1540. INMA has also launched several projects, in cooperation with foreign partners, including the United States, Switzerland, Netherlands, Norway, Germany, and Jordan, to enhance Iraq's capacity to detect and respond to man-made and naturally occurring biological threats.

INITIATIVES TO STRENGTHEN BIOSECURITY IN IRAQ

In 2012, INMA proposed the creation of a high-level interagency coordinating body to create a comprehensive National Biorisk Management System in Iraq. INMA worked with the Public Health Directorate of the Ministry of Health to identify institutions that should be represented on this committee and to determine the committee's goals for strengthening Iraq's preparedness for natural and man-made disease outbreaks. On May 1, 2012, the General Secretariat of the Council of Ministers issued order No.18778 which established the National Biorisk Management Committee (NBMC). The NBMC is chaired by the Ministry of

Health and is composed of representatives from the following ministries: Agriculture, Defense, Interior, Higher Education, Finance, Planning, Industry, Prime Minister's Council, Science and Technology, Environment, Prime Minister's Advisory Commission, Trade, and Water Resource and Irrigation. Additional ministries participate as is necessary.

The NBMC has identified four priorities for strengthening Iraq's national capacity to counter biological threats: establishing a national pathogen list, building laboratory capacity, developing the capability to conduct joint law enforcement–public health investigations, and establishing a biorisk management law. The NBMC has established sub-committees charged with developing new policies and programs to achieve these four objectives.

National Pathogen List

The NBMC is leading the development of a comprehensive list of human, plant, animal, and zoonotic pathogens that will serve multiple purposes in Iraq's National Biorisk Management System. The national pathogen list will become part of the system regulating the import, export, and transfer of dual-use materials. In addition, the list will be used to determine appropriate biosafety and biosecurity measures that laboratories will need to implement. Finally, the list will be used to guide bioterrorism preparedness and response activities. A subcommittee of the NBMC, which is composed of INMA, the Ministry of Public Health, and the Ministry of Agriculture, has worked on developing the list since the beginning of 2014. The subcommittee has used pathogen lists developed by other countries and international organizations, including the United States, European Union, and Australia Group, as a starting point for its work. However, these lists are shaped by national security, public health, agricultural, and economic factors unique to each country and organization. For example, foot-and-mouth disease (FMD) is viewed as a top bioterrorist threat by the United States due to the high vulnerability of its cattle to this disease and the severe economic consequences that an FMD outbreak would have (3). By contrast, FMD is endemic in Iraq and to cope with recurrent major FMD outbreaks, Iraq has developed a robust veterinary and vaccine response strategy (4). Therefore, imposing tight biosecurity limits on FMD virus would not make sense for Iraq. Instead of incorporating an existing pathogen list directly into its National Biorisk Management System, the subcommittee has sought to identify key criteria used to develop these lists and adapt them to Iraq's unique situation. During the spring of 2015, one of the authors served as an CRDF Global Iraqi Bioscience Fellow at George Mason University where he evaluated the criteria used by the United States and European countries to assess and rank the safety and security risks posed by different pathogens. Based on this evaluation and other biorisk assessment exercises, the NBMC is considering applying the following criteria to generate its national pathogen list: pathogenicity, mode of transmission, route of exposure, level of morbidity and mortality, degree of antibiotic resistance, local availability of effective treatments and preventive measures, local diagnostic capabilities, potential economic losses caused by animal and plant agents, and the potential impact on the health-care system.

Building Laboratory Capacity

A strong public health laboratory system is the foundation for an effective defense against naturally occurring infectious diseases and bioterrorism. The Ministries of Health and Agriculture maintain networks of laboratories to conduct surveillance, detection, and diagnosis of human, animal, and plant disease outbreaks (5). These ministries operate laboratories at biosafety level 1 and 2, which limits Iraq's capability to safely conduct diagnostic tests on viruses such as highly pathogenic H5N1 avian influenza (6). Since 2009, the INMA has engaged in cooperative programs with Switzerland and the United States to rebuild Iraq's public health laboratory capacity with the ultimate goal of establishing a biosafety level 3 laboratory. Iraqi officials and scientists from the Ministries of Agriculture, Health, and Science and Technology have received training on biosafety, biosecurity, emergency preparedness and response, and biorisk mitigation. Iraq has received assistance from the United States in the areas of disease surveillance, detection, diagnosis, biosafety, and biosecurity. To guide this work, INMA established an interagency technical working group to review Iraq's biosurveillance capabilities, identify gaps, and propose solutions. The United States is in the process of upgrading the equipment at Iraqi public health and veterinary labs and linking provincial-level labs into a nation-wide network to improve Iraqi biosurveillance capabilities for human and animal disease outbreaks. This initiative helps Iraq meet its commitment under the 2005 International Health Regulations to develop core capacities in laboratory services and disease surveillance. In addition, the United States has provided some Iraqi laboratories with upgrades to their physical security, including the installation of electronic door locks to prevent unauthorized access to pathogen collections. Areas in need of further investment include improving biological waste management systems at public health labs, developing education and training curriculum, standardizing laboratory protocols, and developing an indigenous capability to maintain and repair biosafety equipment.

Public Health–Law Enforcement Investigation

The potential for terrorists to employ CBRN weapons poses new challenges to both law enforcement and public health. Of all of the CBRN threats, biological weapons are the most difficult to investigate because of the potentially long delay between when an attack occurs and when the government becomes aware that an attack has taken place. Since it can be difficult to initially determine if a suspicious outbreak is natural or deliberate, public health authorities need to know when and how to contact law enforcement agencies if they suspect an outbreak is not natural. Likewise, law enforcement agencies need to be aware of the types of information that could indicate a terrorist group is interested in, has acquired, or has released biological agents and have procedures for sharing such information with public health authorities. Once it has been confirmed that an attack has occurred, public health and law enforcement agencies will need to coordinate their investigations to ensure that they collect and share the right information in a timely manner (7). The Ministry of Interior

(MoI), which is in charge of investigating acts of terrorism, does not have a history of working with either the Ministry of Health or Ministry of Agriculture. Although MoI has extensive experience dealing with conventional improvised explosive devices and explosive ordnance disposal, it does not have any capacity for dealing with CBRN weapons or contaminated evidence. INMA is working to establish a joint public health–criminal investigative capacity in Iraq by holding interagency meetings, workshops, and training sessions to develop plans and procedures for information sharing and investigation in the event of a CBRN incident.

Biorisk Management Law

Iraq lacks a legal framework for regulating the full range of biological risks, including disease outbreaks caused by natural sources, terrorists, and laboratory accidents, which threaten Iraq's health and security. The NBMC has formed a subcommittee headed by INMA to draft a new law to provide a legal umbrella for the implementation of biorisk management policy in Iraq, including the national pathogen list, associated biosafety and laboratory security regulations, and the roles and responsibilities of different Iraqi ministries in strengthening Iraq's preparedness for natural and deliberate disease threats. The NBMC's goal is to issue the new law within 2–3 years.

CONCLUSION

Since 2004, Iraq has made significant progress in improving its capacity to prevent the proliferation of CBRN weapons and their means of delivery in accordance with its legal, constitutional, and treaty obligations. Iraqi efforts to strengthen its preparedness for natural and deliberate disease threats has received valuable assistance from foreign partners who have engaged in collaborative capacity building activities over an extended period of time. A major challenge facing Iraq is the need to sustain these capabilities over time, especially if foreign assistance declines. Iraq will need to continue making investments in infrastructure, information technology, and human capital to ensure that its biosafety, biosecurity, and biosurveillance systems remain effective. The INMA has played a key role in Iraq's development of a National Biorisk Management System, but achieving the four objectives established by the NMBC will require further interagency cooperation among Iraqi ministries as well as continued support from Iraq's foreign partners. Only by working together can Iraq and the international community reduce the risks posed by infectious diseases, bioterrorism, and the misuse of biology.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

MA was funded by CRDF Global through the Iraqi Biosciences Fellowship Program.

REFERENCES

1. Republic of Iraq. *Sharing of Experiences, Lessons Learnt and Effective Practices, in the Areas Covered by Resolution 1540 (2004)*. Report Submitted to the UNSCR 1540 Committee (2014). Available from: http://www.un.org/en/sc/1540/pdf/Iraq%20Letter%20re%20effective%20practices%202014.pdf
2. Iraq. *The Implementation of the Biological Weapons Convention and Disease Monitoring in Iraq*. Geneva, Switzerland: Working Paper BWC/MSP/2009/MX/WP.27 (2009).
3. Breeze R. Agroterrorism: betting far more than the farm. *Biosecur Bioterrorism* (2004) **2**:251–64. doi:10.1089/bsp.2004.2.251
4. Mahdi AJ. *Foot and Mouth Disease in Iraq: Strategy and Control*. MS thesis, Kansas State University (2010). Available from: http://krex.k-state.edu/dspace/bitstream/handle/2097/4620/AliMahdi2010.pdf?sequence=1&isAllowed=y
5. Iraq. *Assistance and Cooperation Requirements for Strengthening Disease Monitoring Capacities in Iraq*. Geneva, Switzerland: Working Paper BWC/MSP/2009/MX/WP.8 (2009).
6. Iraq. *Disease Surveillance, Detection, Diagnosis, and Containment in Iraq*. Geneva, Switzerland: Working Paper BWC/MSP/2009/MX/WP.28 (2009).
7. Federal Bureau of Investigation and Centers for Disease Control. *Criminal and Epidemiological Investigation Handbook* (2015). Available from: https://www.fbi.gov/about-us/investigate/terrorism/wmd/criminal-and-epidemiological-investigation-handbook

Conflict of Interest Statement: MA was a Visiting Fellow at George Mason University during the Spring 2015 semester on a CRDF Global Iraqi Bioscience Fellowship. GK has no conflict of interest to declare.

frontiers in Public Health

ORIGINAL RESEARCH
published: 13 October 2015
doi: 10.3389/fpubh.2015.00231

Implementation of the International Health Regulations (2005) through cooperative bioengagement

*Claire J. Standley, Erin M. Sorrell, Sarah Kornblet, Julie E. Fischer and Rebecca Katz**

Global Health Security Program, Department of Health Policy and Management, Milken Institute School of Public Health, The George Washington University, Washington, DC, USA

Cooperative bioengagement efforts, as practiced by U.S. government-funded entities, such as the Defense Threat Reduction Agency's Cooperative Biological Engagement Program, the State Department's Biosecurity Engagement Program, and parallel programs in other countries, exist at the nexus between public health and security. These programs have an explicit emphasis on developing projects that address the priorities of the partner country as well as the donor. While the objectives of cooperative bioengagement programs focus on reducing the potential for accidental or intentional misuse and/or release of dangerous biological agents, many partner countries are interested in bioengagement as a means to improve basic public health capacities. This article examines the extent to which cooperative bioengagement projects address public health capacity building under the revised International Health Regulations and alignment with the Global Health Security Agenda action packages.

Keywords: International Health Regulations, Global Health Security Agenda, biological threat reduction, cooperative bioengagement, health systems strengthening

OPEN ACCESS

Edited by:
Nathan Wolfe, Metabiota, USA

Reviewed by:
Michael Jeroen Adjabeng, Ghana Health Service, Ghana
Ben Oppenheim, New York University, USA

***Correspondence:**
Rebecca Katz
rlkatz@gwu.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *19 June 2015*
Accepted: *28 September 2015*
Published: *13 October 2015*

Citation:
Standley CJ, Sorrell EM, Kornblet S, Fischer JE and Katz R (2015) Implementation of the International Health Regulations (2005) through cooperative bioengagement. Front. Public Health 3:231. doi: 10.3389/fpubh.2015.00231

INTRODUCTION

The concept of "cooperative threat reduction" (CTR) was introduced in the years immediately following the collapse of the former Soviet Union (FSU), when concerns abounded that the equipment, expertise, and materials used as part of state-supported nuclear programs were suddenly vulnerable to exploitation and misuse (1). To counter this apparent threat, Senators Nunn and Luger spearheaded the passage of legislation to form US government programs that would engage the newly formed nations of the FSU in rebuilding peaceful, civilian research, and development capabilities while also reducing the threat that nefarious state or non-state actors would gain access to capabilities for producing an unconventional weapon. From the first Department of Defense programs to assist in safely dismantling the Soviet nuclear arsenal authorized by The Soviet Nuclear Threat Reduction Act of 1991 (commonly known as the Nunn-Lugar Act) (2), CTR programs expanded to encompass collaborative efforts by the Departments of Defense, State, and Energy to secure nuclear, chemical, and biological threats throughout the FSU. After the fall of Saddam Hussein's regime in 2003, the State Department recognized the similar need to engage Iraqi scientists who had previously been employed in state-run weapons programs, and thus CTR programs expanded beyond the FSU. CTR has become a global enterprise, with more than $1 billion in US funds annually supporting partnerships in countries on every continent (3, 4).

A hallmark of modern CTR programs is the recognized need to develop projects that meet partner country needs and priorities as well as the goal of reduced threat of misuse or proliferation (5, 6). In the last decade, the CTR framework with respect to biological agents shifted from "cooperative biological threat reduction" to "cooperative biological engagement," or bioengagement, reflecting the transition away from destruction of biological munitions and related manufacturing capabilities to an emphasis on prevention. There are also some key distinctions from parallel chemical and radio-nuclear programs. First, most high-priority biological agents occur naturally in the environment. These pathogens cause disease outbreaks that can severely affect public health and development, and economic stability, and may be difficult to distinguish from an intentional attack without further investigation. Cooperative bioengagement efforts to date have focused on prevention of acquisition from the environment, along with developing enhanced security and safety at laboratories, an emphasis on responsible conduct of research, and improved capacities to detect disease outbreaks and other unusual events (7, 8). These areas of focus aim at limiting the opportunities for accidental or intentional release of a pathogen as well as improving the likelihood that the US and the broader international community would be alerted quickly of any suspicious outbreak. Because many of the biological agents deemed high risk for weaponization by the US and others are also high-priority endemic or epidemic-prone diseases of public health significance, cooperative bioengagement can provide a mutually acceptable platform for forming new country partnerships, particularly in vulnerable or insecure regions or countries where a traditional "security" program might be politically sensitive or operationally limited.

Cooperative bioengagement allows projects to be developed that meet both public health and security objectives, providing incentives for partner countries who often have health and development concerns foremost in their national priorities. These priorities may even be driven by international legal obligations. For example, the World Health Organization's revised International Health Regulations (IHR) mandate that all 196 States Parties develop the core capacities needed to detect, assess, report, and respond to events that could constitute a public health emergency of international concern (PHEIC). By June 2014, the end date of the first 2-year extension for implementation, only 64 countries out of the 196 States Parties to the IHR declared that they had met these minimum core capacity requirements (9). Those States Parties that are not yet in compliance have until June 2016 to develop the necessary capacities, indicating significant opportunity for partnership with the international community, provided objectives of both sides are aligned.

Recognizing the potential benefits of multi-sectoral collaboration with respect to controlling disease outbreaks, in February 2014, 29 countries, together with WHO, the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (OIE) announced the launch of the Global Health Security Agenda (GHSA). While not binding, the GHSA represents a partnership of now over 40 countries committed to accelerating and elevating progress toward "a world safe and secure from infectious disease threats" (10).

In this paper, we sought to identify existing efforts on the complementarity between health security frameworks (11, 12), and explore the extent to which IHR and GHSA overlap with priorities for developing and executing bioengagement programs. Given cooperative bioengagement's emphasis on country partnership and interest in human and animal pathogens, we hypothesized at least some alignment with IHR and GHSA; indeed, through a descriptive mapping exercise and a series of case studies, we demonstrate that cooperative bioengagement provides significant, although imperfect, alignment with these existing health security frameworks.

MATERIALS AND METHODS

Using open source material, we mapped cooperative bioengagement priorities, based on four examples of bioengagement programs, against the core capacity indicators for the IHR and GHSA action packages. We then identified three projects, funded by bioengagement programs, to use as case studies to examine how these efforts aligned with public health capacity building priorities, opportunities for greater cooperative programing, and the potential challenges to achieving cooperative bioengagement aims through the lens of IHR and GHSA.

Cooperative Bioengagement Priorities

Using open source material, we first sought to characterize common elements across different biological engagement programs. Programs were selected using the following criteria:

- Information on objectives and program mission were available online
- The program is driven by a security and/or non-proliferation mandate.

Using these criteria, we identified the following four programs to analyze for common themes:

- U.S. Defense Threat Reduction Agency Cooperative Biological Engagement Program (CBEP)
- U.S. Department of State Biosecurity Engagement Program (US BEP)
- United Kingdom Biological Engagement Program (UK BEP)
- Canadian Global Partnership Program (GPP).

As a framework for identifying common mission elements between these programs, we used the three "pillars" identified by CBEP as categories for their programmatic activities, which are "biosafety and biosecurity capacity building," "disease surveillance, detection, diagnosis, and control" (sometimes referred to as "biosurveillance"), and "cooperative biological research" (7, 13).

Mapping Health Security Frameworks Against Bioengagement Pillars

The IHR (14) are a legally binding agreement on health security issues for all Member States of the World Health Assembly, and a key framework against which to map bioengagement priorities. In addition, given its high political profile in the health security community since its launch in 2014, we also selected the GHSA,

a framework intended to promote accelerated implementation of IHR, and other supporting health security frameworks for analysis.

For IHR, we mapped each of the eight core capacities, the four specific hazards, and Points of Entry against cooperative bioengagement priorities, assessing the content of each based on the indicators and attributes contained within the IHR Core Capacity Monitoring Framework (2013) (15). For GHSA, we examined each of the 11 action packages, and their corresponding targets and measures (16), to identify elements to map back to the three bioengagement pillars. **Figure 1** demonstrates the elements of bioengagement, IHR, and GHSA that were used in the mapping process. We arrayed all of these elements in a matrix to facilitate comparison.

Case Studies in Bioengagement

To demonstrate how the elements of IHR and GHSA map to the bioengagement priorities, we examined three case studies. We selected these case studies to be descriptive illustrations of bioengagement programs, and thus was a convenience sample representing each of the three pillars, and with sufficient publicly available information to identify project focus areas and goals. We used open source and online material to describe the following bioengagement projects:

- Development of Uganda's Biosecurity Policy and Bill
- Launch of the Republic of Kenya's Zoonotic Disease Unit (ZDU)
- Iraq Science Fellowship Program (ISFP).

For each case study, stated project objectives were categorized per three bioengagement pillars. The project objectives were also examined in terms of their alignment with IHR core capacities and GHSA action packages.

RESULTS

Bioengagement Priorities

The programmatic focus areas, for each of the four bioengagement programs analyzed (CBEP, US BEP, UK BEP, and GPP), were generally well characterized by the three proposed pillars of "biosafety and biosecurity capacity building," "disease surveillance, detection, diagnosis, and control," and "cooperative biological research." Elements that were not well captured by these categories but were expressed as objectives of the four bioengagement programs included an emphasis on engagement and programmatics in certain countries and regions [including explicit geographic prioritization by "threat" although the criteria for determining level of threat are not always clearly defined (17)]; a focus on adherence to global standards and norms, including the Biological and Toxins Weapons Convention (BWC) (18, 19); and interest in projects relating to bioethics or addressing dual-use research of concern (DURC) (18). **Table 1** outlines the main programmatic focus areas we identified for each program, categorized by bioengagement pillar.

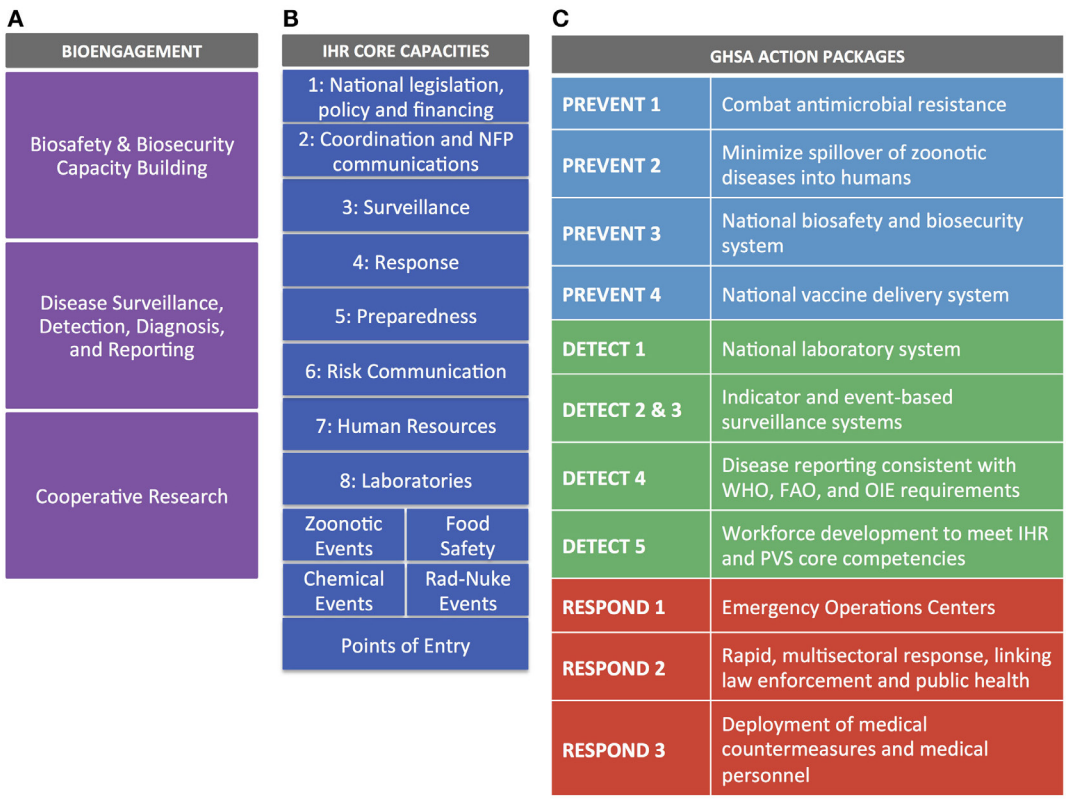

FIGURE 1 | Visual representation of the bioengagement pillars (A); IHR core capacities (B); and Global Health Security Agenda (GHSA) action packages (C) included in the mapping exercise.

TABLE 1 | Bioengagement programmatic efforts and categorization into "pillars" corresponding to "Biological Safety and Security," "disease surveillance, detection, diagnosis, and reporting," and "cooperative biological research."

Program	Biological safety and security	Disease surveillance, detection, diagnosis, and reporting	Cooperative biological research	Other stated priorities?
US CBEP (13, 34)	Consolidation and security of dangerous pathogen collections	Improved capabilities to detect, diagnose, and report outbreaks	Engage scientists in health security research	
	Safety and security of biological facilities		Collaborative research to detect biothreats	
US BEP (17, 35, 36)	Risk assessment	Improved detection and control of priority diseases	Scientist engagement	Explicitly prioritized by threat
	Laboratory security upgrades	Field epidemiology training	Joint scientific collaborations	Reinforce global norms (i.e., BWC)
	Biorisk management training	Surveillance for priority diseases	Research that advances health security	Sustainability
	Biosafety associations		Training and research grants	
	"Holistic" biosecurity (i.e., law enforcement)			
GPP (19, 37)	New lab and facility upgrades	New lab and facility upgrades	Scientist redirection	Guidelines and standards
	Biosafety associations	Diagnostic training		Non-proliferation initiatives
	Biosafety/ security training			
UK BEP (18, 38, 39)	Safety and security training packages	Molecular diagnostics training	Redirection of former weapons scientists	BTWC awareness and implementation
	Biosafety associations	Laboratory capacity building	Collaborative research	Dual use and bioethics training
	Physical security and inventory			

US CBEP, U.S. Defense Threat Reduction Program Cooperative Biological Engagement Program; US BEP, U.S. Department of State Biosecurity Engagement Program; GPP, Canadian Department of Foreign Affairs, Trade, and Development Global Partnership Program; UK BEP, United Kingdom Ministry of Defense Biological Engagement Program.

Bioengagement Priorities and Alignment with IHR and GHSA

Figure 2 illustrates the alignment of IHR core capacity attributes and GHSA action package targets, organized against the pillars of bioengagement. Through this exercise, we came away with three major findings:

(1) Extensive overlap of priorities exists with respect to capacity building for disease surveillance, detection, diagnosis, and control. These capabilities are a priority across both IHR and GHSA frameworks as well as a stated priority for all four bioengagement programs that we included in our analysis. However, it is important to note that most of the bioengagement programs qualify support for this focus area by noting that projects must focus on "priority" pathogens. Similarly, under the "Detect" action packages, GHSA measures the ability to perform surveillance and appropriate diagnostic tests for a limited number of priority syndromes and diseases, while allowing flexibility in determining those priorities. IHR's emphasis is solely on events that could constitute a public health event of international concern, so while certain epidemic-prone diseases are highlighted in Annex 2 (14) as always requiring notification to WHO (or at least requiring critical evaluation of whether notification is necessary), the core capacities needed to identify such events must, by definition, be capable of detecting and reporting all outbreaks.

(2) Significant overlap exists between IHR, GHSA, and bioengagement under the biosafety and biosecurity capacity building pillar, but the range of activities varies. Within the IHR Monitoring Framework, Core Capacities 1 (National legislation, policy, and financing) and eight (Laboratories) address separate elements of this pillar: the ability to develop and implement legislation or regulations and laboratory biosafety and biosecurity, respectively. This latter indicator also includes policies or regulations required above the institutional level. However, IHR does not emphasize the importance of inventory and physical security of pathogens, readily provide a forum for engaging biosafety associations, nor explicitly cover non-diagnostic/clinical settings. GHSA's Prevent 3 action package (biosafety and biosecurity) is more comprehensive, covering all facilities handling especially dangerous pathogens and including pathogen security as a key part of the target (but not a measure of implementation success). While implied, Prevent 3 lacks an explicit mention of using a risk assessment-driven approach to biosafety and biosecurity, which is mentioned as a priority under several bioengagement programs. Under Respond 2 (Linking Public Health with Law Enforcement), GHSA touches on concepts related to "holistic" biosecurity stated in US BEP's program priorities (17) though the bioengagement emphasis is on creating public health and law enforcement linkages to prevent an attack, whereas GHSA is more focused on the response element.

(3) Cooperative biological research is a pillar that does not present any clear alignment with the IHR core capacity indicators nor the GHSA action package targets and measures. As such, our analysis indicated that as defined by most programs, cooperative biological research would not provide a means for partner countries to achieve any aspects of IHR or GHSA compliance. This does not preclude activities that do further IHR and GHSA aims from simultaneously engaging biological scientists in

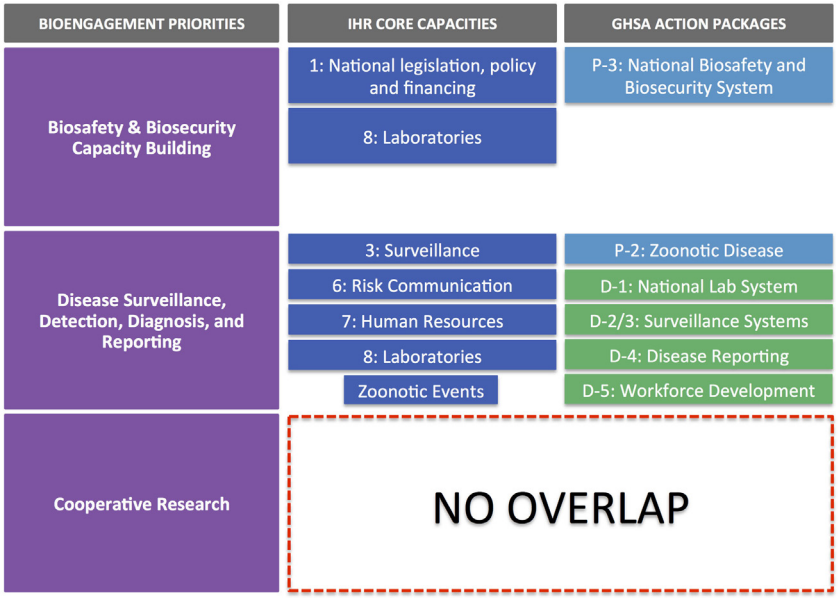

FIGURE 2 | IHR core capacities and GHSA action packages mapped against the three pillars of bioengagement programs. Note that there is no overlap of IHR and GHSA core capacities and action packages with the "cooperative biological research" pillar of bioengagement.

a similar fashion to that achieved by designated cooperative biological research projects; however, the form of the engagement and the way the project is developed are likely to be significantly different from a traditional research project, where the emphasis is on hypothesis-driven investigation, rather than relationship building, training, or applicability to public health.

Cooperative Bioengagement in Action: Case Studies

In order to further explore the extent of overlap between bioengagement priorities and the IHR/GHSA frameworks, we selected three case studies, each representing one of the three main pillars of bioengagement. Through this process, we were able to confirm some of the observations from the *a priori* mapping exercise, while also identify other observations related to the opportunities and challenges of aligning bioengagement programs with implementation of health security frameworks.

Uganda Biosecurity Policy and Bill

In 2012, the Uganda National Council for Science & Technology (UNCST), in collaboration with Global Implementation Solutions (GIS) and with funding support from the US BEP, held a consultative workshop to discuss the development of a new national biosecurity policy (20, 21). The intention was to create a biosecurity policy that would build on and complement Uganda's existing 2008 biotechnology and biosafety policy, and specifically address biosecurity issues as well as Uganda's obligations under the BWC. Since the initial kick-off meeting, there have been a number of additional consultations and sensitization efforts, involving broad representation from Ugandan government ministries as well as a variety of other stakeholders. A Bill, written based on the policy, will be submitted to the Ugandan Parliament by the end of 2015 (21).

Based on stated objectives from UNCST, the Bill will cover seven main objectives (**Table 2**). These outcomes span preparedness, early detection of disease threats, and integrated response to emerging events as well as a recognition of the importance of collaborations an partnerships; these, thus, reach beyond "pure" biosecurity concerns, and touch on aspects of both other pillars of bioengagement. It is, therefore, worth highlighting the distinction between the deliverable of the funded project, which is the development of the Policy and the Bill and its submission to Parliament, versus the projected impact of implementation of the Bill once passed into law. **Figure 3** examines the sequential impact of the development of the Policy and Bill (solid boxes) versus those areas that will also be addressed once the Bill is implemented (transparent boxes).

Overall, the focus of the Policy and Bill development project align very closely with GHSA Prevent 3, with respect to creating a national framework for biosafety and biosecurity (**Figure 3**). There is less alignment with IHR; however, actual implementation of the Policy and Bill will address IHR core capacities, such as Core Capacity 1 (National legislation, policy, and financing) and Core Capacity 8 (Laboratories) and therefore Policy and Bill development can be seen to be acting in support of IHR.

Kenya Zoonotic Disease Unit

The Kenya ZDU was launched in August 2012 as a joint initiative between the then Ministry of Public Health and Sanitation

[now the Ministry of Health (MoH)] and the then Ministry of Livestock Development [now the Ministry of Agriculture, Livestock, and Fisheries (MALF)] (22). Support for the effort, including construction of the building housing the unit and the development of the strategic plan, were provided by CBEP and the US BEP (23), with technical assistance from GIS, a US BEP grantee (24). Both programs continue to support ZDU activities, including the drafting of guidelines for priority zoonotic diseases and a regional One Health conference hosted by the ZDU (24, 25).

TABLE 2 | Characterization of Uganda Biosecurity Policy stated outcomes within the defined pillars of bioengagement programs.

Uganda Biosecurity Policy stated outcomes (20)	Bioengagement pillar
Ensure emergency preparedness, at the field, community, and health facility levels	Disease surveillance, detection, diagnosis, and control
Facilitate early detection of and response to emerging disease threats	Disease surveillance, detection, diagnosis, and control
Ensure integrated response to threats and rationalization of controls	N/A
Put in place the containment principles, technologies, and practices that are implemented, to prevent the unintentional exposure to pathogens and toxins, or their accidental or intentional release	Biosafety and biosecurity capacity building
Reduce the risk of biothreats by guiding the development of safety and security standards that are consistent with international guidelines and requirements	Biosafety and biosecurity capacity building
Create opportunities for capacity building to generate a critical mass of scientific and technological expertise in biorisk management	Biosafety and biosecurity capacity building
Promote collaborations, partnerships, and linkages at national, regional, and international levels to provide inclusive, effective, affordable, and practical solutions to pressing local and international concerns	Cooperative biological research

The goals for the ZDU, as described in the 2012–2017 Strategic Plan, can be summarized as improving surveillance and control of zoonotic diseases; establishing partnerships related to One Health; and conducting research on zoonotic pathogens (**Table 3**). These align closely with the disease surveillance and cooperative research pillars of bioengagement.

In terms of alignment with IHR and GHSA objectives, the main overlaps are with core capacities/action packages associated with surveillance, reporting, and zoonotic diseases (**Figure 4**). It is also worth noting that the targets and measures outlined for surveillance (Detect 2/3) under GHSA do not specifically cover zoonotic diseases; suggested syndromes are provided within the text of the action package, but the choice should be based on country priorities, therefore providing an opportunity to include priority zoonotic syndromes. As seen earlier, the cooperative biological research elements of the ZDU goals do not directly correspond to any IHR core capacity indicator or GHSA action package.

Iraq Science Fellowship Program

The ISFP was founded in 2008 as a mechanism for creating opportunities for Iraqi scientists to further their careers and create new collaborative opportunities by spending 3–6 months at a U.S. institution while conducting a specific research project. US CTR programs and the UK BEP have both supported biological

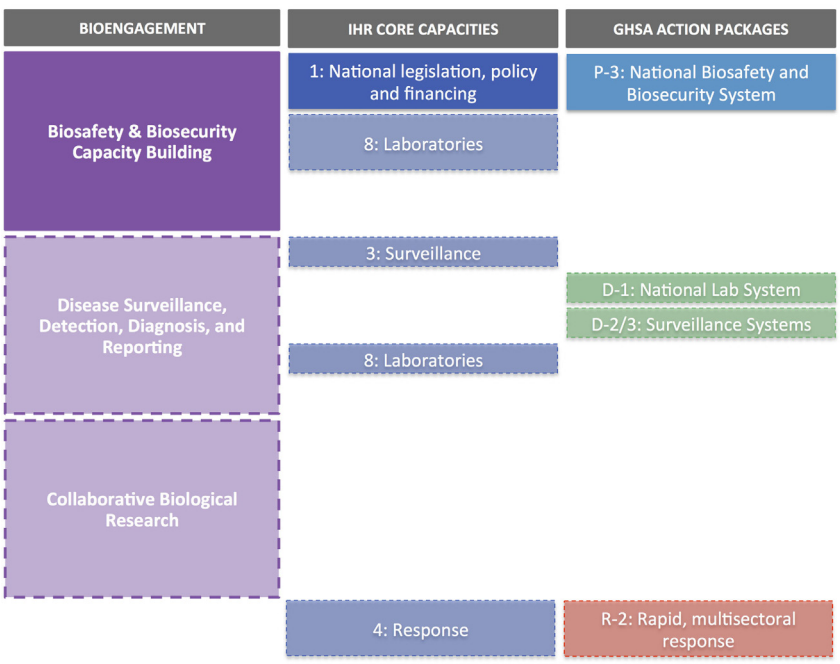

FIGURE 3 | Uganda Biosecurity Policy and Bill case study. Solid boxes indicate elements supported directly by the development of the Policy and Bill; transparent boxes indicate additional elements which would be addressed through implementation of the Bill (and after approval by the Ugandan Parliament).

ISFP fellows in past years (26), and in 2014, CBEP also launched a biologist-specific fellowship program [known as the Iraq Biosciences Fellowship Program (IBFP)] supporting scientists from the Iraq MoH, Ministry of Agriculture, and Ministry of Science and Technology (26).

The stated objectives of ISFP and IBFP relate entirely to creating research networks, building scientific capacity, and providing the fellows with skills to advance their research careers, all of which fall under bioengagement's cooperative biological research pillar, and particularly the focus areas related to scientist engagement (**Table 4**). However, an examination of the scientific disciplines given selection preference by IBFP (27) indicate that beyond these stated objectives, there may in fact be significant additional overlap with bioengagement priorities. For example, a scientist selected to work on biosecurity policy issues at a US institution would not only constitute cooperative research but also biosecurity capacity building; likewise a fellow working on novel viral diagnostic methods would also be addressing biosurveillance objectives while part of the collaborative research effort.

Taken at face value, given that neither IHR nor GHSA explicitly contain indicators or measures related to cooperative research or scientist engagement, there is little if any overlap between ISFP/IBFP and these health security frameworks (**Figure 5**). However, the specific research projects conducted by the researchers participating in the fellowship programs may themselves relate back to biosafety, biosecurity, or disease surveillance efforts, which in turn could have bearing on IHR or GHSA implementation. To our knowledge, lists of ISFP and IBFP fellowship projects are not published online or available through open sources, so we were unable to determine if additional areas of alignment exist between ISFP/IBFP and IHR or GHSA at the research project level.

DISCUSSION

This work describes the alignment of cooperative bioengagement programmatic elements with IHR core capacities and GHSA action packages, as described through mapping of program priorities and qualitative examination of three case studies. Overall, IHR and GHSA represent opportunities to bioengagement programs that may be seeking leverage points around which to form new partnerships, and also to the implementation community, who may be able to better tailor projects and receive funding from bioengagement programs to support existing health security activities that advance IHR and GHSA compliance. These areas, notably aspects of biosafety and biosecurity capacity building and biosurveillance, could also provide an opportunity for

TABLE 3 | Characterization of the goals of the Republic of Kenya Zoonotic Disease Unit (per the 2012–2017 Strategic Plan) within the defined pillars of bioengagement programs.

Kenya Zoonotic Disease Unit goals (2012–2017)	Bioengagement pillar
To strengthen surveillance, prevention, and control of zoonoses in both humans and animals	Disease surveillance, detection, diagnosis, and control
To establish structures and partnerships that promotes One Health approaches	Disease surveillance, detection, diagnosis, and control
To conduct applied research at the human–animal–ecosystem interface in order to better understand the mechanism of maintenance and transmission of zoonotic pathogens	Cooperative biological research

TABLE 4 | Characterization of the goals and expected outcomes of the Iraq Science Fellowship Program within the defined pillars of bioengagement programs.

Iraq Science Fellowship Program goals and outcomes (26, 40)	Bioengagement pillar
Enrich their scientific knowledge	Cooperative biological research
Develop valuable skills to promote Iraq's scientific community	Cooperative biological research
Learn new methods and expertise to improve research capabilities	Cooperative biological research
Opportunity to increase Iraq's scientific capacity	Cooperative biological research

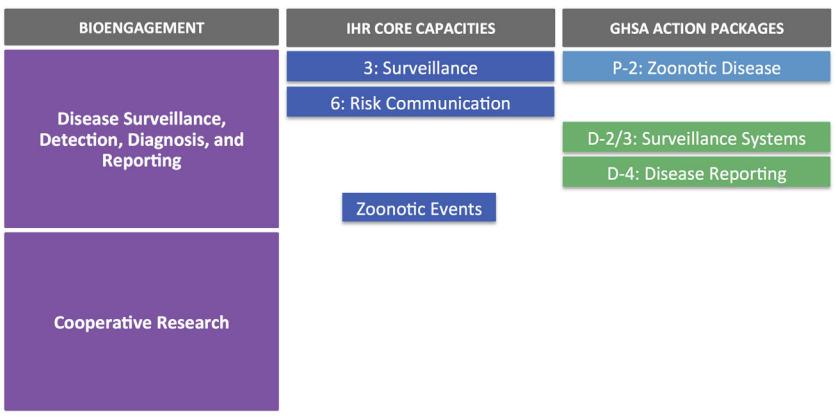

FIGURE 4 | Republic of Kenya Zoonotic Disease Unit case study.

FIGURE 5 | Iraq Science Fellowship Program and Iraq Biosciences Fellowship Program case study. Note that without greater detail on the research projects supported through these fellowships, it is difficult to determine the elements of IHR or GHSA that might be advanced through the projects; IHR and GHSA otherwise do not contain elements that align directly with collaborative research aims or scientist engagement.

bioengagement programs to develop evaluation measures and metrics that meet partner country targets with respect to IHR and GHSA.

However, we also observed clear gaps in the extent of overlap between some areas of bioengagement efforts and IHR/GHSA priorities. Within the biosecurity pillar in particular, there remain differences related to definitions of key terms, ideal outcomes, and the precedence placed on national-level legislative and regulatory frameworks as a mechanism to achieve sustainable implementation. For example, while IHR's biosafety and biosecurity-related attributes are focused primarily at the level of diagnostic laboratories, the emphasis of GHSA's Prevent 3 action package is on the development of national-level regulatory or legislative frameworks, thus targeting a different level of decision-makers and stakeholders; the Uganda Biosecurity Policy and Bill project, which we selected as our biosecurity case study, fit more closely with this latter definition. Similarly, within the veterinary sector, "biosecurity" usually does not refer to preventing unauthorized access to pathogens, but rather corresponds to the suite of control measures applied to protect against and prevent the spread of disease (28). In addition, words, such as "security" and "biosecurity," may be associated with cultural and social sensitivities, which may differ significantly between partner countries and regions, and thus need to be taken into account when describing project outcomes and establishing new partnerships. The Kenya ZDU case study highlighted that although bioengagement programs have a strong interest in zoonotic diseases, it may prove more challenging to link zoonotic disease-focused bioengagement projects with IHR or GHSA-driven targets and measures.

The cooperative biological engagement pillar did not have clear areas of alignment with any of the IHR core capacities or GHSA action packages. There is no mention of networking between researchers or scientists, or the basic research and development underpinnings of health security in IHR or GHSA. However, when examining the case studies, it was clear that projects funded under different pillars that explicitly address IHR and GHSA objectives can also have a clearly defined research component; likewise, "pure" cooperative research projects, if focused on subject areas that related to disease surveillance, control, or biorisk management, may also result in improved IHR or GHSA compliance in the long-term. One solution to this apparent paradox could be to reconsider the three pillars of bioengagement, and rather than consider cooperative research as an end in itself, recognize it as a means for advancing sustainable capacity building for biosafety/biosecurity and biosurveillance activities. Another option could be to categorize programmatic efforts based on the target audience: i.e., national-level initiatives, facility-level initiatives, and science/knowledge-based initiatives. There is precedence for both such approaches (7, 8), and so future academic analyses of the overlaps between bioengagement and other frameworks may want to examine the impact of these alternative characterizations. Such an approach might also address the observation from the Uganda Biosecurity Bill case study that there can be a distinction between the direct outcomes of the funded project (in this case, a Policy and piece of legislation) and future projected impacts (implementation of the legislation). Adapting the way projects are characterized within bioengagement programs might provide a more explicit means for acknowledging the broad benefits that can sometimes accrue from even narrowly focused projects.

The case studies revealed several other points for further consideration. When researching case studies, we found little information on specific bioengagement-funded projects in the public domain, let alone on project outcomes, which significantly limited the extent to which we could analyze alignment with IHR/GHSA. To our knowledge, there are no universally accepted metrics consistently used across bioengagement programs; those that have been developed for specific programs, such as RAND's effort related to DTRA's CBEP (7), have not been used publicly to evaluate project success, but rather may be kept for internal use within the program. While it is possible to conduct convincing meta-analyses without shared metrics, the lack of outcome-focused data in the public domain at all related to these programs limits opportunities for such analysis. Implementers can moreover have legal restrictions on the extent to which they can publish project-related information (for example they may be constrained by Non-Disclosure Agreements), or may feel obliged not to publicize project details if they feel it could jeopardize future funding. Moreover, some implementers, particularly those with a public health mandate, may not be comfortable advertising their funding as coming from a bioengagement source due to the security connotations. Overall, these factors make it very challenging for independent, objective analysis of bioengagement programs and the extent to which they are successful at promoting compliance with health security frameworks.

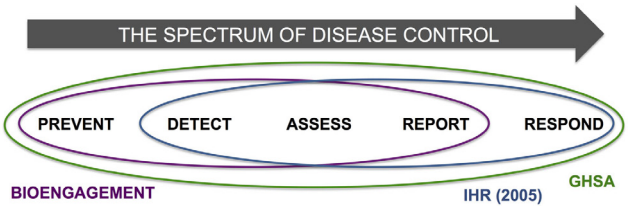

FIGURE 6 | Schematic demonstration of the spectrum of disease control, incorporating the steps of "Prevent," "Detect," "Assess," "Report," and "Respond," and highlighting the areas of each encapsulated by bioengagement, IHR, and GHSA priorities and efforts.

A final observation, which was largely beyond the scope of this paper but should be examined in more detail in further analytical efforts, was to note the aspects of IHR and GHSA that might be overlooked if bioengagement programs were the only groups working in a particular country or region. Thinking across the spectrum of disease control, it is notable that bioengagement programs focus primarily on prevention, detection, assessment, and reporting of disease events, rather than response; IHR and GHSA, in contrast, place a high priority on response capabilities, through core capacity 4 (Response) and action package Respond 1–3, respectively (**Figure 6**). While bioengagement programs have made significant investments in building emergency operations capacities in several countries [examples include Jordan (29), Vietnam (30), and Uganda (31)], these have been framed exclusively as "preparedness" efforts, and particularly among the US-funded bioengagement programs, funding is generally not available to support activities that are solely response-oriented, given their non-proliferation and prevention mandate (32). This suggests that countries seeking to form partnerships to develop their capacities to respond to disease threats may need to look outside the bioengagement donor community, or describe their needs carefully to emphasize the preparedness elements.

This descriptive exercise provided an opportunity to acknowledge the diversity of programmatic efforts throughout different international bioengagement programs, and also recognize the potential for greater alignment with parallel efforts that exist solely in the global health sphere, including those which have yet to become significant stakeholders in the on-going health security dialog. Major players in global health, for example, "traditional" vertical disease control programs, such as the President's Emergency Plan for AIDS Relief (PEPFAR), and even non-communicable disease control efforts, are realizing their role in meeting some aspects of IHR implementation, for example in building laboratory capacity (33). Some of these efforts may moreover directly align with bioengagement priorities, even if the motivation behind the project differs. The opportunity to re-examine the donor landscape with respect to the full spectrum of disease and biological threats may, thus, provide opportunities for greater coordination between sectors. This, in turn, could have a positive impact not only on project outcomes, but also for in-country perceptions of bioengagement programs, leading in turn to deeper, more sustainable relationships with partner countries.

AUTHOR CONTRIBUTIONS

CS and ES conceived the concept; CS, ES, SK, JF, and RK conducted the research; CS drafted the manuscript, with technical input from ES, SK, JF, and RK; all authors reviewed and approved the final version.

ACKNOWLEDGMENTS

The authors would like to acknowledge the invaluable assistance provided by Andrea Vaught (Department of Health Policy and Management, Milken Institute School of Public Health) in the formatting and finalization of the manuscript. We are grateful for the comments and suggestions of two anonymous reviewers, whose input strengthened the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpubh.2015.00231

REFERENCES

1. Bernstein PI, Wood JD. *The Origins of Nunn-Lugar and Cooperative Threat Reduction*. Washington, DC (2010). Available from: http://ndupress.ndu.edu/Portals/68/Documents/casestudies/CSWMD_CaseStudy-3.pdf
2. *Soviet Nuclear Threat Reduction Act of 1991*. Washington, DC: The Library of Congress (1991). Available from: http://thomas.loc.gov/cgi-bin/query/C?c102:./temp/~c102rGC8gn
3. Nikitin MBD, Woolf AF. *The Evolution of Cooperative Threat Reduction: Issues for Congress* (2014). Available from: http://www.fas.org/sgp/crs/nuke/R43143.pdf
4. Kane C. *From Donor to Partner: The Evolution of U.S. Cooperative Threat Reduction into Global Security Engagement* (2011). Available from: http://www.nti.org/analysis/articles/donor-partner-evolution-us-cooperative-threat-reduction-global-security-engagement/
5. *Fiscal Year 2015 Budget Estimates: Cooperative Threat Reduction Program* (2014). Available from: http://comptroller.defense.gov/Portals/45/Documents/defbudget/fy2015/budget_justification/pdfs/01_Operation_and_Maintenance/O_M_VOL_1_PART_2/CTR_PB15.pdf
6. Committee on Strengthening and Expanding the Department of Defense Cooperative Threat Reduction Program, Committee on International Security and Arms Control, Policy and Global Affairs. *Global Security Engagement: A New Model for Cooperative Threat Reduction*. Washington, DC: The National Academies Press (2009). Available from: http://www.nap.edu/catalog/12583/global-security-engagement-a-new-model-for-cooperative-threat-reduction
7. Young S, Willis HH, Moore M, Engstrom J. *Measuring Cooperative Biological Engagement Program (CBEP) Performance: Capacities, Capabilities, and Sustainability Enablers for Biorisk Management and Biosurveillance*. Santa Monica, CA: (2014). Available from: http://www.rand.org/content/dam/rand/pubs/research_reports/RR600/RR660/RAND_RR660.pdf
8. Kuntz C, Salerno R, Jacobs E. *A Biological Threat Prevention Strategy: Complicating Adversary Acquisition and Misuse of Biological Agents*. Lanham, MD: Center for Strategic and International Studies (2013). Available from: http://csis.org/files/publication/130503_Kuntz_BioThreatPrevention_Web.pdf
9. *WHA reaches agreement on polio, International Health Regulations and strengthening surgical care*. Geneva: World Health Organization (2015). Available from: http://www.who.int/mediacentre/news/releases/2015/wha-22-may-2015/en/

10. *The Global Health Security Agenda: United States Department of Health and Human Services* [cited 2015 June 4]. Available from: http://www.globalhealth.gov/global-health-topics/global-health-security/ghsagenda.html
11. Bakanidze L, Imnadze P, Perkins D. Biosafety and biosecurity as essential pillars of international health security and cross-cutting elements of biological nonproliferation. *BMC Public Health* (2010) **10**(Suppl 1):S12. doi:10.1186/1471-2458-10-S1-S12
12. Katz R, Sorrell EM, Kornblet SA, Fischer JE. Global health security agenda and the international health regulations: moving forward. *Biosecur Bioterror* (2014) **12**(5):231–8. doi:10.1089/bsp.2014.0038
13. Katz RL, Lopez LM, Annelli JF, Arthur RR, Carroll D, Chapman LW, et al. U.S. Government engagement in support of global disease surveillance. *BMC Public Health* (2010) **10**(Suppl 1):S13. doi:10.1186/1471-2458-10-S1-S13
14. World Health Organization. *International Health Regulations (2005)*. Geneva: World Health Organization (2008). Available from: http://whqlibdoc.who.int/publications/2008/9789241580410_eng.pdf
15. World Health Organization. *IHR Core Capacity Monitoring Framework: Checklist and Indicators for Monitoring Progress in the Development of IHR Core Capacities in States Parties*. Geneva: World Health Organization (2013). Available from: http://apps.who.int/iris/bitstream/10665/84933/1/WHO_HSE_GCR_2013.2_eng.pdf
16. *Global Health Security Agenda: Action Packages* (2014). Available from: http://www.cdc.gov/globalhealth/security/pdf/ghsa-action-packages_24-september-2014.pdf
17. *U.S. Department of State Biosecurity Engagement Program. Implementing Partners Workshop* (2014).
18. *UK Biological Engagement Programme. Strengthening Biological Security* (2013). Available from: http://www.slideshare.net/MOD_CDE/uk-biological-engagement-programme-cde-themed-competition-l
19. Ellis M. *Strengthening Global Biological Security. Global Partnership Program* (2011). Available from: http://www.pbphpc.org/wp-content/uploads/2011/07/SGBS-Washington.pdf
20. Onapa MO, Jjagwe R. *Formulation of a National Biosecurity Regulatory Framework for Uganda* (2015). Available from: http://www.uncst.go.ug/component/content/article/29/217-formulation-of-a-national-biosecurity-regulatory-framework-for-uganda.html
21. *Biosecurity Policy Development: Global Implementation Solutions (GIS)* [cited 2015 June 19]. Available from: http://www.gisworldwide.org/#!about1/c20hi
22. *About ZDU: Republic of Kenya Zoonotic Disease Unit* (2015) [cited 2015 June 19]. Available from: http://zdukenya.org/about-zdu/
23. Republic of Kenya Zoonotic Disease Unit. *Introduction of the Kenya Zoonotic Disease Unit* (2013). Available from: http://zdukenya.org/wp-content/uploads/2013/02/Introduction-of-the-Kenya-Zonnotic-Disease-Unit.pdf
24. *GIS One Health Activities: Global Implementation Solutions (GIS)* [cited 2015 June 19]. Available from: http://www.gisworldwide.org/#!one-health/ctw8
25. *Regional Conference on Zoonotic Diseases in Eastern Africa*. Republic of Kenya Zoonotic Disease Unit (2015) [cited 2015 June 19]. Available from: http://zdukenya.org/regional-conference-on-zoonotic-diseases/
26. *Current Funding Opportunities: 2015 Iraq Science Fellowship Program*. CRDF Global (2014) [cited 2015 June 19]. Available from: http://www.crdfglobal.org/grants-and-grantees/current-funding-opportunities/2014/05/27/2015-iraq-science-fellowship-program
27. *Current Funding Opportunities: 2014-2015 Iraq Biosciences Fellowship Program*. CRDF Global (2014) [cited 2015 June 19]. Available from: http://www.crdfglobal.org/grants-and-grantees/current-funding-opportunities/2014/01/15/2014-2015-iraq-biosciences-fellowship-program
28. Kelly AM. Veterinary medicine in the 21st century: the challenge of biosecurity. *ILAR J* (2005) **46**(1):62–4. doi:10.1093/ilar.46.1.62
29. *International Emergency Preparedness Team*. U.S. Centers for Disease Control and Prevention (2014) [cited 2015 June 19]. Available from: http://www.cdc.gov/globalhealth/healthprotection/ghsb/iept/
30. Osius T. *Opening Remarks at the Inauguration of the Vietnam Emergency Operations Center*. U.S. Department of State (2015). Available from: http://vietnam.usembassy.gov/ambspeech-020315.html
31. Kennedy D. *CDC Strategic Health Engagements in Africa: Capacity Building for the Future*. U.S. Centers for Disease Control and Prevention (2015). Available from: http://www.geisglobalevents.org/Home/AFRICOM_2015_PostConference
32. U.S. Department of State Bureau of Counterterrorism. *Chapter 4: The Global Challenge of Chemical, Biological, Radiological, or Nuclear (CBRN) Terrorism*. U.S. Department of State (2013). Available from: http://www.state.gov/j/ct/rls/crt/2013/224827.htm
33. Specter S, Schuermann L, Hakiruwizera C, Sow MS. ASM LabCap's contributions to disease surveillance and the International Health Regulations (2005). *BMC Public Health* (2010) **10**(Suppl 1):S7. doi:10.1186/1471-2458-10-S1-S7
34. *CTR Biological Threat Reduction*. U.S. Defense Threat Reduction Agency [cited 2015 June 19]. Available from: http://www.dtra.mil/Missions/Partnering/CTRBiologicalThreatReduction.aspx
35. *Bureau of International Security and Nonproliferationo – GTR Program. U.S. Foreign Assistance Performance Publication: Fiscal Year 2009*. U.S. Department of State and U.S. Agency for International Development. Available from: http://www.state.gov/documents/organization/159277.pdf
36. *Biosecurity Engagement Program: U.S. Department of State Biosecurity Engagement Program* [cited 2015 June 19]. Available from: http://bepstate.net/
37. *Biological Weapons Non-Proliferation Programming under Canada's Global Partnership Program*. Canada Department of Foreign Affairs and International Trade (2005). Available from: http://www.partnershipforglobalsecurity-archive.org/Documents/vestergaard_presentation.pdf
38. Department of Energy and Climate Change. *Global Threat Reduction Programme: Biological Non-Proliferation Programme*. UK Government (2013). [cited 2015 June 19].
39. *Global Threat Reduction Programme: Eighth Annual Report 2010*. United Kingdom Foreign & Commonwealth Office, Department of Energy & Climate Change, and Ministry of Defence (2011). Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/48038/1361-gtrp-eighth-annual-report.pdf
40. *U.S.-Iraq Partnership Strengthens Iraqi Scientists*. CRDF Global (2013). Available from: http://www.crdfglobal.org/news-and-events/press-room/news/u.s.-iraq-partnership-strengthens-iraqi-scientists

Conflict of Interest Statement: Rebecca Katz is Principal Investigator (PI) on one project funded by DTRA CBEP. Julie E. Fischer is PI on two US BEP-funded grants. Erin M. Sorrell, Sarah Kornblet, Julie E. Fischer, and Rebecca Katz receive salary support from DTRA CBEP and US BEP projects; Claire J. Standley receives salary support from the DTRA CBEP project only. Erin M. Sorrell and Claire J. Standley previously served as contractors within US BEP through February 2014 and August 2014, respectively.

frontiers in Public Health

ORIGINAL RESEARCH
published: 07 December 2015
doi: 10.3389/fpubh.2015.00264

Building International Genomics Collaboration for Global Health Security

Helen H. Cui, Tracy Erkkila, Patrick S. G. Chain and Momchilo Vuyisich*

Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA

Genome science and technologies are transforming life sciences globally in many ways and becoming a highly desirable area for international collaboration to strengthen global health. The Genome Science Program at the Los Alamos National Laboratory is leveraging a long history of expertise in genomics research to assist multiple partner nations in advancing their genomics and bioinformatics capabilities. The capability development objectives focus on providing a molecular genomics-based scientific approach for pathogen detection, characterization, and biosurveillance applications. The general approaches include introduction of basic principles in genomics technologies, training on laboratory methodologies and bioinformatic analysis of resulting data, procurement, and installation of next-generation sequencing instruments, establishing bioinformatics software capabilities, and exploring collaborative applications of the genomics capabilities in public health. Genome centers have been established with public health and research institutions in the Republic of Georgia, Kingdom of Jordan, Uganda, and Gabon; broader collaborations in genomics applications have also been developed with research institutions in many other countries.

Keywords: genomics, next-generation sequencing, international collaboration, pathogen detection, global health, one health, bioinformatics, capability development

OPEN ACCESS

Edited by:
Nathan Wolfe, Metabiota, USA

Reviewed by:
Paras Jain, Albert Einstein College of Medicine, USA
Eric Delwart, Blood Systems Research Institute, USA

****Correspondence:***
Helen H. Cui
hhcui@lanl.gov

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *14 August 2015*
Accepted: *06 November 2015*
Published: *07 December 2015*

Citation:
Cui HH, Erkkila T, Chain PSG and Vuyisich M (2015) Building International Genomics Collaboration for Global Health Security. Front. Public Health 3:264. doi: 10.3389/fpubh.2015.00264

BACKGROUND AND INTRODUCTION

In early 2010, the prominent general scientific society in the US, the American Association for the Advancement of Science (AAAS), recognized that international scientific collaboration is critical to addressing complex societal challenges in health, agriculture, environment, energy, and global security, and organized a series of four conferences under the common theme of "International Engagement: Responsible Bioscience for a Safe and Secure Society." In a period of 2 years, these conferences examined the critical issues and explored the potentially sustainable approaches for collaboration between the scientists in the US and other countries, with the focus on the broader Middle Eastern and North African region. The major themes resulting from these conferences revealed a clear desire for cooperative research and development with responsible scientific practice and progressive action to enhance the infectious disease surveillance under the One Health concept (1). Although the AAAS conference series was focused on one region, the concept derived from the meeting and approaches that can be taken are undoubtedly applicable to many other countries and regions. Successful and sustainable partnerships that build upon mutual interests, complementary capabilities, and supporting infrastructure are essential to addressing complex global challenges, such as approaching the One Health concept. Scientists with interest and resources to initiate and

maintain partnerships with colleagues in other countries, and who are able to overcome existing barriers to collaboration, serve as the foundation for successful international cooperation.

Reducing global health security risk from the spread of dangerous infectious diseases, whether natural or manmade, is a shared priority among the worldwide public health communities. It has also become an overarching objective for international cooperative biothreat reduction and scientific engagement efforts. Engaging with and empowering infectious disease detection and surveillance capabilities in the partner countries enable a global network to reduce risk and enhance compliance with international guidelines, such as the International Health Regulations (from the World Health Organization in 2005) and those by World Organization for Animal Health (OIE). Extensive international collaborations have been developed to address global health security challenges, with activities, such as bioethics discussion, responsive scientific conduct, biorisk management, and field epidemiology, which have resulted in a positive global impact.

A well-suited scientific approach complementary to the above activities is next-generation sequencing-enabled genomics research for global health security applications. Genomics is a relatively new scientific discipline but is fundamental to many approaches to health security. Aided by highly automated Next-Generation DNA Sequencing (NGS) technologies, the field has been advancing dramatically, both in the depth of understanding genome structure and function of living organisms, and in the breadth of applications in areas, such as medicine (disease mechanisms, diagnostics, and therapeutics) and agriculture (2, 3). Using high-throughput DNA sequencing for pathogen detection during an infectious disease outbreak or for biosurveillance was previously slow, cost prohibitive, and required in-depth training and expertise. In the past decade, due to the advancement of highly automated instrument platforms, streamlined operational procedures and protocols, available reagent kits, and data analysis pipelines (4), NGS has been in effect democratized, expanding from central sequencing laboratories to individual institutions. As a result, genomics has become a highly desirable area that inspires broad international collaborations targeting diverse applications (5). Examples of global health security related applications include infectious disease diagnosis, previously unknown pathogens identification, sample archive characterization, and biosurveillance. Even though NGS technology has made high-throughput DNA sequencing more accessible, the existence of the automated instruments and streamlined procedures cannot replace the in-depth knowledge and expertise to use the instruments with high proficiency and accuracy; nor can the analytic algorithms analyze the experimental outcomes with precise interpretation. Without specialized training and technical expertise, these technology advancements cannot be fully utilized.

In support of the overarching scientific engagement objectives, the Genome Science Program at the Los Alamos National Laboratory (LANL) has been leveraging our own capabilities to provide support to a growing number of partner countries on four continents in developing molecular genomic-based pathogen detection and characterization capabilities. We approach such development by first understanding partner country needs and gaps in building genomics and bioinformatics capacities. This is followed by scientific and technical training, facility building, and dissemination of pipelines and processes for microorganism genotypic characterization. Continuous subject matter expertise reachback support is provided to the collaborators. Our efforts in these areas enable the education of the next generation of life scientists in the partner countries, providing a robust foundation in genomic science through didactic and practical instruction, and the required technical infrastructure for genomics research by integrating sequencing and analytic capabilities.

While these genomics capabilities are being developed, the collaborators start engaging in scientific collaborations utilizing NGS and other molecular techniques, such as real-time PCR and immunoassays. By applying these methods and correlating the findings, we aim to better understand emerging infectious diseases within the partner countries that are of global concern. The collaboration efforts will not only benefit the host countries and regions with state-of-the-art life science methods and technologies but also build a trusted international network with a shared passion in addressing global emerging infectious disease challenges. Such networks provide the potential for sharing resources, which is essential for approaching the One Health objective and reducing health threats globally.

STRATEGY AND APPROACH

Our international genomics development and collaboration efforts have been built upon prior engagement activities carried out by other institutions and sponsored by various donors. We initially sought collaborations with countries that have been partners with our sponsors for several years, such as the Republic of Georgia and the Kingdom of Jordan. Many of the collaborating institutions already have well-established capabilities in molecular diagnostics and genetic analysis, and hold principle responsibilities in public and veterinary health in their respective countries. To effectively achieve the scientific engagement objectives, we have developed a phased strategy for establishing genomics laboratories in partner countries, followed by continuous technical support and cooperative scientific research to address some of the pressing public and veterinary health challenges.

Phased Approaches for Capability Building

Our phased approach for establishing genomics centers with partner countries provides both the starting momentum to build a capability and flexibility that is responsive to the evolution of technologies and business practices, which reduces risks in achieving sustainability. The four phases can be summarized as: (1) scientific engagement evaluation, (2) technical strategy and development planning, (3) infrastructure and technical capability building, and (4) instrument operation and bioinformatics training. One most critical consideration throughout all phases is the sustainability development. Depending on the existing capabilities and infrastructure readiness, we do not necessarily go through all phases with any given partner institution.

The phased approach to engagement and sustainment is shown in **Figure 1**.

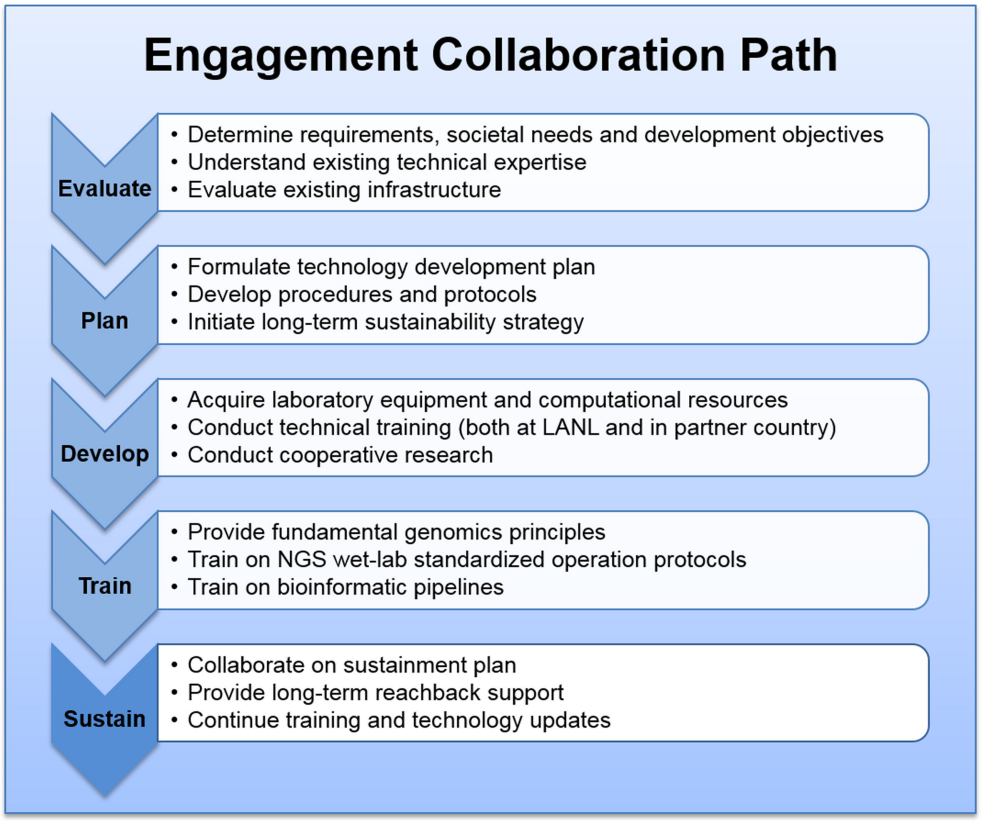

FIGURE 1 | Phased approach, for NGS-based scientific engagement development.

Phase I, Scientific Engagement Evaluation and Requirement Analysis

In order to provide meaningful assistance to our partner countries and their particular institutions, it is essential to understand host country public health challenges and requirements, and to carefully consider what a genomics capability can accomplish to address their specific challenges. We first work directly with host nation scientists and leadership to understand existing capabilities and expertise in pathogen detection and characterization, urgent public and veterinary health issues, and to assess the technologies and capabilities compatible with genomics and bioinformatics. Essential areas of consideration include: host country objectives in genomic science and technology, desired near- and long-term outcomes, type and availability of samples to be processed, anticipated throughput and turnaround, potential future resources, anticipated implementation timeline, and a long-term sustainability vision. Other assessment areas include the existing technical expertise in laboratory operation and bioinformatics, laboratory infrastructure, computational infrastructure, and administrative skills. An initial development strategy is formulated based on a requirement analysis for the engagement partner to achieve full operational capability, including the approach to narrowing the gaps between the desirable end goals and the current status of these resources.

Direct interactions with prominent regional life scientists, medical, public health, and agriculture professionals provide the collaborators with unique opportunities to identify areas for advancement that would significantly impact the science in the region or in the country; this allows us to build trust for a long-term collaborative relationship. The availability of advanced genomic science capabilities meets the foundational need that would impact almost all areas of the life sciences and presents opportunities for technology and economic development. During this phase, we start deliberating a long-term sustainability plan, based on the initial observations and development objectives.

Phase II, Technical Strategy and Development Planning

Once the requirements and objectives are defined, we begin formulating the technology strategy and development plan for a given institution to reach the target objectives. Planning includes personnel professional development, infrastructure preparation, NGS instrument acquisition, and initiating collaboration logistics including sample transportation and information sharing procedures. A technical action plan is generated that provides a framework of recommended protocols, workflows, and required training. In addition, the action plan provides recommended paths to address the gaps and challenges in the existing laboratory equipment, facility, and computational resources. We also generate a training plan to outline the tasks required for laboratory staff to obtain proficiency using specific operational protocols, pipelines, and processes. This includes sample preparation and management, instrument operation, sequence data analysis, and initial interpretation for biological significance.

During this phase, we also introduce fundamental principles and basic techniques of molecular- and genomic-based approaches for pathogen and infectious disease detection and characterization, and assist partner countries to further define priorities and needs for enhancing these capabilities. Initial training in these topics is provided either at LANL and/or at the partner site.

Phase III, Infrastructure and Technical Capability Development

The engagement activities subsequently progress into preparation of the NGS laboratory facility, acquisition of laboratory instruments and computational equipment, dissemination of standardized operational protocols, and technical training for proficiency building. The engagement collaborators work closely to design and configure appropriate laboratory space and bioinformatics systems at the partner facility. In this phase, careful consideration of local constraints, logistics, and experience available at the engagement partner site are critical.

The steps in this phase include

1. *Wet-lab and informatics hardware specification and laboratory setup.* The specification is based on the existing facility infrastructure, equipment, expertise and target goals. It includes wet-lab operational protocols and bioinformatics systems required to accomplish the development objectives. Laboratory equipment required for various wet-lab processes, and informatics hardware and software to process data and perform analysis are specified and acquisition is initiated. In some cases, the acquisition is handled by LANL, and in other cases by a third-party integrating contractor. Laboratory setup is performed by a combination of vendors, partner staff, and LANL staff. Good laboratory practice (GLP) is used as a guiding principle to ensure high quality experimental output, minimize potential cross-contamination, and protect the laboratory personnel.
2. *Deploy wet-lab processes.* We work with engagement partners to deploy standardized wet-lab protocols and processes. Example protocols include DNA library preparation for bacterial isolate sequencing, RNA library preparation for viral sample sequencing, targeted microbial sequencing protocols, whole genome sequencing (WGS), and general Illumina MiSeq platform sequencing procedures. Implementation of GLP is stressed during this phase, and training in GLP is delivered throughout all relevant phases.
3. *Deploy informatics pipelines.* Informatics pipelines coordinated with wet-lab protocols are provided to the partner facility. Examples include initial sequencing data quality control and reporting, data quality treatment, genomic assembly, reference-based assembly, and metagenomic analysis.

Phase IV, Training on Instrument Operation and Bioinformatics Processing

A strong foundation built through training is a vital component of the entire engagement activity. The collaborators work closely to coordinate training sessions in both the US and on-site in the host country facility. The primary goal is to instill a strong foundation in both scientific principles and operational processes during the exchange. On-site training focuses on development and implementation of sample preparation, sequencing library generation, genome sequencing, and sequencing data analysis procedures in compliance with GLP regulations. Great attention is paid to hands-on troubleshooting, and additional training is provided as needed. Training in this phase is an avenue to disseminate best biosafety and biosecurity practices.

Throughout the exchange and following period, the US scientists monitor partner performance and provide feedback to the sponsors and partner country scientists. Bioinformatics reachback assistance is provided to support current mission and long-term development of sustainable approaches. Assistance is also provided to the partners in the development and execution of research projects that utilizes the capability. Updating or expanding protocols and processes and providing new versions to the engagement partners are a continuous practice.

Sustainability Development

The sustained utilization of the new genomics capability and continued service to society is the ultimate goal for the genomics centers being developed. To support sustainable development, we assist the institutions in identifying research and operational areas, such as training their fellow scientists and public health practitioners, conducting hypothesis-driven research, and providing services to community. The basic research, applied research, and operational areas that will benefit from advanced genomics science are extremely broad, virtually in all life sciences and related fields of practice. Priorities are identified based on research activities, public health responsibilities, and perceived societal significance. These priority areas include emerging or unknown pathogenic microorganism identification, molecular epidemiology, phylogenetic characterization of infectious agents, and infectious disease surveillance. In addition, genomics-assisted design and development of new diagnostic signatures and assays, and their applications in veterinary and agricultural practices have become important areas of engagement.

After deploying genomics technologies at a partner site and delivering initial training, successful capability establishment at the host site typically requires on going assistance. LANL provides reachback support to the engagement partners, by delivering remote, near real-time technical advice on wet-lab, informatics, and data analysis. Laboratory support includes advice on sample preparation procedures and protocols, and recommendation of specific reagents or kits to use. Informatics-focused reachback support can be especially important for some partners, as they may have limited existing informatics capability to draw on. Reachback on data analysis is very common, and can usually be a point of collaboration, from sequencing data quality evaluation to interpreting biological significance. Internet connectivity is very important for these purposes, and can sometimes be the rate-limiting factor.

Scientists at partner institutions are regularly invited to attend and contribute to the annual Sequencing, Finishing, and Analysis in the Future (SFAF) meetings that LANL has hosted for the past decade [SFAF (6)]. This conference provides an opportunity for the engagement partners to gain up-to-date knowledge on new techniques and technologies, and to present their own research results. Since 2013, LANL has hosted annual NGS

training workshop in Los Alamos for engagement partners. The workshop includes principles of NGS and hands-on training in both laboratory techniques and bioinformatics. These opportunities provide avenues to broaden expertise and our collaborations with international partners.

RESULTS

We have successfully established genome centers with partner institutions in four different countries: Republic of Georgia, Kingdom of Jordan, Uganda, and Gabon, and developed extensive genomic research collaboration with several other countries. These genome centers are provided with a standardized sequencing platform, standard operating protocols and GLP training, and bioinformatics analysis tools and associated computational hardware.

Standardized Platforms and Processes

At LANL, we have extensive experience with most NGS platforms. During the past decade, we have acquired every major NGS instrument to conduct scientific research and to provide sequencing service and training to our collaborators. The Illumina MiSeq instrument was selected as the sequencing platform of choice for the international genomics engagement activities for its compact physical size, accompanying reagent kits, standardized operation procedures, high quality data output, and broad applications (4, 7). We have developed and disseminated standardized protocols for sample preparation, quality control, sequencing, and data management protocols. The standardized protocols require a few other supporting laboratory instruments for DNA fragmentation, quality control, quantification of DNA fragments, and quantitative PCR, which are also provided to our partner facilities. The general process flow is summarized in **Figure 2**.

Standard computer software and hardware have also been specified to simplify installation and ongoing support. For computational resources, the CLC Genomics Workbench (Qiagen) was selected from several commercial options as an analysis and visualization tool. This software package provides an integrated environment with most types of tools necessary for our partners to analyze sequencing data generated by MiSeq. In-house developed bioinformatics tools are also disseminated to the partner laboratories. The LANL developed EDGE Bioinformatics (8) is an analytic tool that integrates a selection of open source tools accessed by a web-based interface. EDGE has been deployed to several of our partner facilities and has proven to be an easy-to-use tool for rapid analysis of NGS data, requiring little specialized bioinformatics expertise. The computational hardware provided to our partners is fully sufficient to process sequencing data generated by the MiSeq platform at the partner's site. This approach to deploying and training on a repertoire of technology and equipment has enabled us to franchise genomics centers with standardized procedures for our partners. The training and support we can provide is greatly enabled by this standardization.

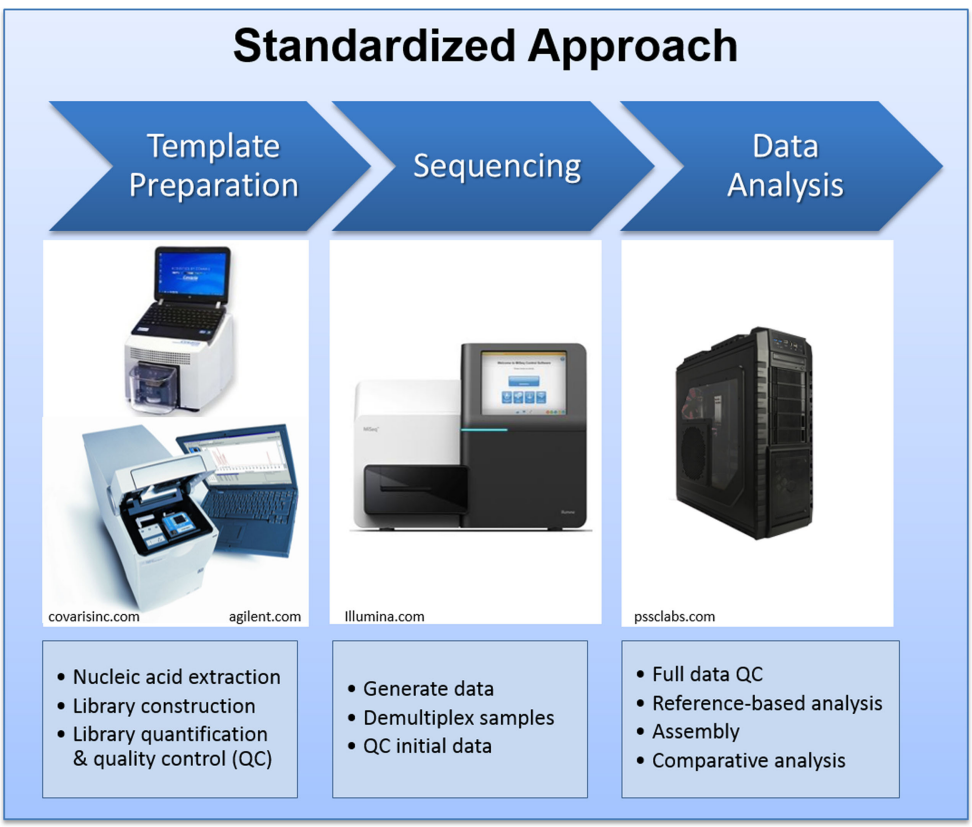

FIGURE 2 | The standard NGS laboratory and bioinformatics pipeline setting deployed to the partner laboratories.

Current Collaborations

We have successfully established genome centers with four partner institutions in Georgia, Jordan, Uganda, and Gabon (**Figure 3**). These genome centers are provided with a standardized sequencing platform, standard operation and GLP protocols, and bioinformatics analysis tools and associated computational hardware. We travel to each site and assist with setup and configuration for laboratory equipment and computational resources. On-site training for both laboratory and bioinformatics are also provided during these visits. We typically guide the partners to perform one or more sequencing runs on the newly provided equipment while we are on-site. A brief description of each genome center is provided in Section "Established Genome Centers" below. In addition to establishing these genome centers, we provide training and engage in collaborative research with other institutions that either already have NGS capability or are on a path to acquire it. This type of collaboration has taken place in nine different countries (**Figure 3**), and allows the partners to broaden international collaboration networks and to exercise and enhance local capabilities. These collaborations bring together complementary skill sets and resources, benefiting all participating parties and paving a good path to improved global health.

Established Genome Centers

Republic of Georgia: Genomics Center at the National Center for Disease Control and Public Health

The National Center for Disease Control and Public Health of Georgia (NCDC) was originally established in 1937 as the Anti-Plague Station, investigating species and the spread of *Yersinia pestis* in Georgia. After a tularemia outbreak in Southern Georgia in 1946, the institutional mission scope was expanded to include the investigation of tularemia and subsequently other infectious diseases, such as anthrax. The institution has since become the leading epidemiological control organization in Georgia, and later established the national reference laboratory now known as the R. G. Lugar Center for Public Health Research in 2013.

The genome center at Georgian NCDC was established in 2012. Extensive training took place for the Georgian scientists at both LANL and NCDC on NGS sample preparation protocols, MiSeq operations, and bioinformatics analysis of sequencing data. Reachback support in the form of teleconference calls, emails, and remote logins has been delivered to the Georgians on a regular basis. Today, the NCDC genome center is fully functional and more than 10 staffs have been trained and are regularly using the capabilities to conduct scientific research in pathogen virulence characterization and biosurveillance for infectious diseases. LANL and NCDC scientists have also embarked on joint research projects utilizing the new NGS capability. An example of research activities is highlighted in Section "Initial Cooperative Research Highlights," and a new project has been initiated as a result of a competitive proposal process.

Kingdom of Jordan: Jordan University of Science and Technology

The Princess Haya Biotechnology Center (PHBC) at the Jordan University of Science and Technology (JUST) was established in 2005. The Center represents the state-of-the-art biotechnology in clinical applications, supported by Princess Haya Bint Al Hussein, with a primary responsibility directed toward supporting the Jordanian medical and scientific communities. It is hosted at the King Abdullah University Hospital and is equipped with modern facilities for carrying out research, diagnostic laboratory testing and training in areas of genomics, proteomics, metabolomics, hematology, and other clinical areas.

In 2012, LANL initiated collaborations with the PHBC to develop an NGS genomics capability. Applying the phased approach detailed in Section "STRATEGY AND APPROACH,"

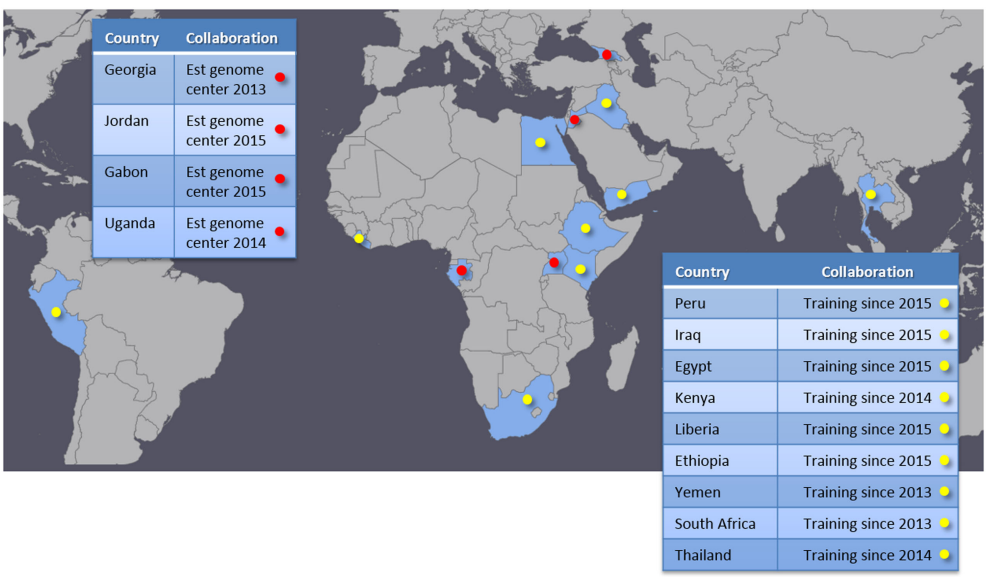

FIGURE 3 | Partner countries. Red marks indicate genome centers established by our program. Yellow marks countries that are in collaborations focusing on training and research.

a functional NGS facility for genomics research and training was established in early 2015. Leveraging our experience with the Georgian project, we specified laboratory equipment and computational resources to match the capability target, level of PHBC staff expertise, and existing PHBC laboratory resources. During 2013 through 2015, extensive training and traveling took place for JUST staff to receive GLP, laboratory protocols, and bioinformatics analysis trainings, at both LANL and JUST. Currently, joint research projects are being planned to exercise the genomics capability and further train JUST staff.

Uganda: The Uganda Virus Research Institute

Uganda Virus Research Institute (UVRI) is a Ugandan national reference laboratory for various viral diseases including hemorrhagic fevers, HIV, and Influenza. An NGS capability based on the MiSeq platform is being incorporated into laboratory operations at UVRI, especially during epidemic outbreak investigations for enhanced pathogen detection.

In August 2013, LANL staff traveled to Uganda for the initial evaluation of UVRI facilities and expertise, the Phase I in our overall approach. New construction at UVRI was beginning on the space that would become the home of the genome center. Recommendations on infrastructure details as a fully functional genomics laboratory were quickly provided and implemented. Detailed planning for the genome center development completed in 2013. During 2014, facility construction was completed and laboratory equipment was delivered. In October 2014, LANL scientists traveled to Uganda to assist UVRI staff in setting up the genome center and to deliver the first round of on-site training to UVRI staff. LANL staff returned to UVRI in February 2015 to deliver additional training in laboratory protocols and bioinformatics analysis. During this visit, the genome center started sequencing and analyzing several clinical samples of unknown viral diseases.

Gabon: International Center for Medical Research in Franceville

Los Alamos National Laboratory is working with a research institute in Gabon, the International Center for Medical Research in Franceville (CIRMF) to enhance their genomics capabilities. CIRMF was founded in 1979 to conduct research in fertility, immunology, pathology, and microbiology. Today, CIRMF has refocused almost exclusively on infectious diseases with an emphasis on microbiological monitoring and assistance to public health. LANL is assisting the CIRMF to set up a genomics center to achieve these objectives. In February 2015, LANL staff traveled to Franceville to provide technical assistance to the facility and staff, to assist in addressing technical challenges in operating a newly acquired MiSeq, and to set up a new bioinformatics system that we assisted CIRMF in acquiring. In this engagement, the early evaluation and planning phases were unnecessary, as the CIRMF was already in the process of developing a genomics capability. An evaluation of the current state and the level of expertise were performed and recommendations for additional equipment and training were given. These recommendations are now being implemented.

Initial Cooperative Research Highlights

Through these international partnerships, extensive collaboration planning and executions are being carried out. After 2–3 years of collaboration, research efforts have started producing initial results at various centers. A few examples are listed below:

CRISPR Analysis of *Yersinia pestis* Strains from Georgia

Three *Y. pestis* strains isolated from two historical natural plague sites of Georgia were analyzed based on their CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) features. In the *Y. pestis* genome, three CRISPR elements YPa, YPb, and YPc are found at three distinct loci and presently 137 spacer sequences are known (9). These CRISPR elements are highly correlated with the region and location of isolation, potentially providing important genotyping and evolutionary information, which could help to trace the source of outbreaks. The MiSeq platform at the Georgian NCDC Lugar Center was used to conduct WGS of the isolates. The Georgian scientists have gained sufficient analytical skills through training, and successfully applied the two bioinformatic pipelines that have been deployed (CLCBio Genomics Workbench and EDGE Bioinformatics) to perform the analysis.

MERS-CoV Patient Sample Analysis

In 2013, an outbreak of MERS-CoV occurred in the Middle East, including Jordan (10, 11). Two individuals with severe respiratory symptoms were suspected of having been infected by the virus were admitted to the King Abdullah University Hospital; the throat swabs were sent to PHBC for analysis. One of the patients did not survive after hospital admission. Total RNA was isolated by PHBC staff, and samples were sequenced with the MiSeq platform and supplemented with additional Illumina HiSeq sequencing. The presence of MERS-CoV virus was confirmed in both samples using NGS. Even though the samples were degraded, the virus was present in sufficient abundance for us to obtain the entire viral genome from one of the samples, revealing that it was related to the original outbreak in Saudi Arabia. This type of high-resolution analysis of clinical samples can now be achieved at JUST by the trained local scientists and laboratory staff. It will enable faster detection and more detailed tracking of future outbreaks, and reach further research objectives.

RNA Virus Sequencing and Analysis at the UVRI Genome Center

After two rounds of training, the UVRI staff analyzed several blood samples from patients presenting with various viral hemorrhagic fever (VHF) symptoms. RNA was extracted and converted to sequencing libraries for sequencing on the UVRI MiSeq instrument. The URVI scientists applied the standardized protocols received during the laboratory setup and training session to perform an initial quality control check, quantification, RNA sample preparation, and concentration measurement. Sequencing library preparation, validation, and quantification were performed using standard protocols provided by LANL, and NGS was performed on the UVRI MiSeq. The Ugandan scientists successfully applied the two bioinformatic pipelines that have been deployed (CLCBio Genomics Workbench and EDGE Bioinformatics) to perform the

sequencing data analysis. While no hemorrhagic fever viruses were present in the samples, the analyses revealed some potential bacterial pathogens that may have caused the symptoms.

Our collaboration partners continue to actively explore the application of NGS to their local needs. Research ideas include the characterization of samples from long-term archives. This would include the application of NGS to the investigation of past unexplained illnesses, characterization of pathogens from geographical areas across different time periods, and possibly the identification of previously unknown pathogens to the local area. Studying the zoonotic diseases endemic to the partner countries is also of high interest. And many partners are interested in developing a quality reference database of pathogen strains within their purview. Application to clinical, animal, and agricultural samples is a common theme.

CONCLUSION

Our experience in collaborating with our international partners has been very fruitful and rewarding. We have successfully assisted in the development of genome centers in four countries: Republic of Georgia, Kingdom of Jordan, Uganda, and Gabon. We have also established long-term partnerships with genomic laboratories in many other countries. Enthusiasm for utilizing NGS at each center continues to build, and several projects have begun. These early projects aim to detect and characterize pathogens from clinical, animal, or environmental samples.

These collaborative experiences have not gone without certain challenges. Some of the challenges have been of a logistical nature, such as acquiring reagents in a timely fashion, which is critical to the success of the research and training. On more than one occasion after reagents were ordered, the items were delivered after the manufacturer listed expiration date due to logistical delays. Proper infrastructure support is another main challenge for NGS capability, including environmental heating, cooling and ventilation. Stable and clean electrical power is very important and not always present. Reliable and efficient Internet access is required for NGS operation; Internet access is improving rapidly in all partner locations, but remains a point of concern.

Partner staff expertise and proficiency building is key to the success of these collaborations. Most scientists and technicians are experienced biologists but lack bioinformatics expertise. Training a biologist in the techniques of genome science is relatively straightforward but achieving informatics proficiency requires a basic starting level which is lacking. The partner countries typically do not have ready access to bioinformatics skills, and this continues to be a challenge. Our recent deployment of the EDGE bioinformatics tool has been an attempt to overcome this challenge, which was designed for general users without specialized bioinformatics training.

Sustainment of the genome science capabilities with our partners is a key concern. We continue to improve the expertise of these partners through training and scientific conference opportunities. Sustainable use of the established NGS technologies will be strengthened by performing regular research projects. These research projects will be funded by international sponsors, and will provide funds for reagents and research time by partner staff. Over time, this approach will enable the partners to exercise and develop genomic capabilities and continue on a path to sustainability. A recently funded research project with NCDC in Georgia resulted from a competitive proposal process is a positive example.

Development of genome centers in many countries around the World will not only help the local public health authorities identify and monitor disease outbreaks but will also enable true global biosurveillance at a high temporal, geographic, and information resolution (12–14). Considering the potential for the rapid spread of the known pandemic pathogens and future emerging ones, a network of genome centers that can rapidly provide high-resolution genomic data will help improve the speed and accuracy of outbreak detection and monitoring, and reduce the global threat from these pathogens. The speed and efficacy of mitigation will be largely increased by the ability to test the samples locally, without having to ship the sample long distances. This approach offers a large safety benefit, as clinical samples containing live pathogens do not have to be moved long distances or across borders, and potentially cause additional outbreaks. Successful scientific partnerships and sustainable technical capacity are essential to addressing complex global health security challenges to realize the One Health concept.

ACKNOWLEDGMENTS

We wish to thank all members of the Los Alamos National Laboratory Genome Science Program staff for their commitment and expertise throughout this development.

FUNDING

The authors wish to thank the US Defense Threat Reduction Agency Cooperative Biological Engagement Program, US Department of State Biosecurity Engagement Program, the United Kingdom Global Partnership Programme, and the Foreign Affairs, Trade and Development Canada Global Partnership Program, for funding the effort.

REFERENCES

1. Berger KM, Husbands J, Sture J, Khan E, Cohen M. *Future Opportunities for Bioengagement in the MENA Region*. New York, NY: AAAS (2013).
2. Green ED, Guyer MS, National Human Genome Research Institute. Charting a course for genomic medicine from base pairs to bedside. *Nature* (2011) **470**:204–13. doi:10.1038/nature09764
3. Relman DA. Microbial genomics and infectious diseases. *N Engl J Med* (2011) **365**(4):347–57. doi:10.1056/NEJMra1003071
4. Mardis ER. Next-generation sequencing platforms. *Annu Rev Anal Chem* (2013) **6**:287–703. doi:10.1146/annurev-anchem-062012-092628
5. Manolio TA, Abramowicz M, Al-Mulla F, Anderson W, Balling R, Berger AC, et al. Global implementation of genomic medicine: we are not alone. *Sci Transl Med* (2015) **7**(290):290ps13. doi:10.1126/scitranslmed.aab0194
6. Los Alamos National Laboratory. *Sequencing, Finishing, and Analysis in the Future Meeting*. (2015). Available from: http://www.lanl.gov/conferences/sequencing-finishing-analysis-future/
7. Illumina. Illumina Sequencing Technology (2015). Available from: https://www.illumina.com

8. EDGE. *EDGE: Empowering the Development of Genomics Expertise.* (2015). Available from: https://edge.readthedocs.org/en/latest/
9. Barros PM, França CT, Lins RH, Santos MD, Silva EJ, Oliveira MB, et al. Dynamics of CRISPR loci in microevolutionary process of *Yersinia pestis* strains. *PLoS One* (2014) **9**(9):e108353. doi:10.1371/journal.pone.0108353
10. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Eng J Med* (2012) **367**:1814–20. doi:10.1056/NEJMoa1211721
11. Coleman CM, Frieman MB. Coronaviruses: important emerging human pathogens. *J Virol* (2014) **88**:5209–12. doi:10.1128/JVI.03488-13
12. Laksanalamai P, Joseph LA, Silk BJ, Burall LS, L Tarr C, Gerner-Smidt P, et al. Genomic characterization of *Listeria monocytogenes* strains involved in a multistate listeriosis outbreak associated with cantaloupe in US. *PLoS One* (2012) **7**:e42448. doi:10.1371/journal.pone.0042448
13. Norelle IS, Porter JL, Seemann T, Watkins A, Stinear TP, Howden BP. Outbreak investigation using high-throughput genome sequencing within a diagnostic microbiology laboratory. *J Clin Microbiol* (2013) **51**(5):1396–401. doi:10.1128/JCM.03332-12
14. Gilmour MW, Graham M, Domselaar GV, Tyler S, Kent H, Trout-Yakel KM, et al. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *BMC Genomics* (2010) **11**:120. doi:10.1186/1471-2164-11-120

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 16 October 2015
doi: 10.3389/fpubh.2015.00239

Comparative proteomic studies of *Yersinia pestis* strains isolated from natural foci in the Republic of Georgia

*Maia Nozadze[1,2], Ekaterine Zhgenti[1,3], Maia Meparishvili[2], Lia Tsverava[2], Tamar Kiguradze[2], Gvantsa Chanturia[3], Giorgi Babuadze[3], Merab Kekelidze[3], Lela Bakanidze[3], Tatiana Shutkova[3], Paata Imnadze[3], Stephen C. Francesconi[4], Richard Obiso[5] and Revaz Solomonia[1,2]**

[1] Institute of Chemical Biology, Ilia State University, Tbilisi, Georgia, [2] I.Beritashvili Center for Experimental Biomedicine, Tbilisi, Georgia, [3] National Center for Disease Control, Tbilisi, Georgia, [4] Naval Medical Research Center, Frederick, MD, USA, [5] Attimo Research and Development, Blacksburg, VA, USA

OPEN ACCESS

Edited by:
Nathan Wolfe,
Metabiota, USA

Reviewed by:
Shivani Agarwal,
Northwestern University, USA
Stephan Patrick Willias,
University of Florida, USA

***Correspondence:**
Revaz Solomonia
revaz_solomonia@iliauni.edu.ge

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: 17 June 2015
Accepted: 02 October 2015
Published: 16 October 2015

Citation:
Nozadze M, Zhgenti E, Meparishvili M, Tsverava L, Kiguradze T, Chanturia G, Babuadze G, Kekelidze M, Bakanidze L, Shutkova T, Imnadze P, Francesconi SC, Obiso R and Solomonia R (2015) Comparative proteomic studies of Yersinia pestis strains isolated from natural foci in the Republic of Georgia. Front. Public Health 3:239. doi: 10.3389/fpubh.2015.00239

Yersinia pestis, the causative agent of plague, is a highly virulent bacterium responsible for millions of human deaths throughout history. In the last decade, two natural plague foci have been described in the Republic of Georgia from which dozens of *Y. pestis* strains have been isolated. Analyses indicate that there are genetic differences between these strains, but it is not known if these differences are also reflected in protein expression. We chose four strains of *Y. pestis* (1390, 1853, 2944, and 8787) from the National Center for Disease Control and Public Health collection for proteomic studies based on neighbor-joining tree genetic analysis and geographical loci of strain origin. Proteomic expression was analyzed using two-dimensional gel electrophoresis and mass spectrometry. Select *Y. pestis* strains were grown under different physiological conditions and their proteomes were compared: (1) 28°C without calcium; (2) 28°C with calcium; (3) 37°C without calcium; and (4) 37°C with calcium. Candidate proteins were identified and the differences in expression of F1 antigen, tellurium-resistance protein, and outer membrane protein C, porin were validated by Western blotting. The *in vitro* cytotoxicity activity of these strains was also compared. The results indicate that protein expression and cytotoxic activities differ significantly among the studied strains; these differences could contribute to variations in essential physiological functions in these strains.

Keywords: *Yersinia pestis*, proteome, 2-D gel electrophoresis, virulence, Republic of Georgia

INTRODUCTION

Yersinia pestis is a Gram-negative, rod-shaped bacterium that is the causative agent of plague, a high-mortality disease recorded throughout history. Most human *Y. pestis* infections are flea-borne and manifest as bubonic plague; the other two forms of plague, septicemic and pneumonic, are rare. Public health experts recognize plague as a re-emerging infectious disease. In addition, *Y. pestis* carries the potential for use as a biological weapon because it can be mass-produced, is easily

aerosolized, and may result in highly fatal pneumonic plague, which can spread person-to-person via inhalation of infectious air-borne droplets (1, 2).

Strains of *Y. pestis* isolated from different natural foci exhibit distinct biochemical and virulent phenotypes (1); laboratory passaging is also known to influence the accumulation of genetic polymorphisms in plague vaccine strains (3). In the event of infectious disease outbreaks, detailed knowledge of the molecular characteristics of *Y. pestis* strains is important to enable epidemiological traceability.

Several factors are thought to contribute to the persistence of *Y. pestis* in the environment. The stability of the rodent-flea infection cycle allows for constant circulation of the organism within susceptible host species (4). In this repeating cycle of rodent-flea infection, the organism is capable of causing fatal disease in Muridae and Sciuridae populations, and fleas must continually infect new hosts to find new blood meals (5). *Y. pestis* is therefore dependent on vectors and reservoirs for continued enzootic replication.

The longevity of fleas infected with *Y. pestis* also contributes to the duration of the enzootic cycle of infection; these insect reservoirs can sustain enzootic foci over long periods before their death, even when the host rodents are asymptomatic (6). In addition, the longevity of certain species of rodents post-infection can play a role in enzootic cycles of infection: certain rodent species, including some within the *Microtus* (7–10) and *Meriones* (11–14) genera, exhibit moderate- to high-resistance to plague and can carry *Y. pestis* without quickly dying (15). Some isolates of *Y. pestis* are less virulent than others, and this decreased virulence is thought to contribute to the stable enzootic circulation of *Y. pestis* because host and reservoir live longer and therefore have more opportunities to transmit the organism (16). In ancient plague foci, such as those in the Caucasus, vector–host cycles with different transmission and disease dynamics may co-exist (17) in which *Y. pestis* variants are highly virulent in certain rodents but avirulent in man (16).

Plague re-emergence in areas where the disease has been absent for many decades is not well understood (18–20). Two different mechanisms have been proposed: either *Y. pestis* continues circulating in animal populations at undetectable levels, or it is reintroduced to the animal population (21, 22). Although *Y. pestis* evolved from the enteric bacterium *Yersinia pseudotuberculosis*, which can survive for long periods in soil and water, selective pressure exerted by vector-borne transmission has resulted in the loss of such functionality (18, 23, 24). *Y. pestis* lacks the survival traits of other yersiniae, and long-term persistence outside of a host or vector is unlikely.

Plague epidemics have been recorded throughout the last century in the Republic of Georgia. During the last 50 years, two natural foci have been described from which multiple *Y. pestis* strains have been isolated (3). These foci are located in the Transcaucasian highland (encompassing the Ninotsminda and Akhalkalaki regions on the Javakheti plateau) and the Iori region (encompassing eastern Georgia and the border between Georgia and Azerbaijan) (25).

Yersinia pestis was isolated for the first time in Georgia in the spring of 1966, coinciding with a rodent epizootic of plague in several foci within the country. The cultures were isolated from the carcasses of *Meriones libycus* (red gerbils) and their fleas, *Xenopsylla conformis* and *Ceratophyllus laeviceps*. Strains were isolated from Eldari, Nazarlebi, and Taribani steppes, and the existence of a natural focus of plague among a Georgian population of *M. libycus* was confirmed for the first time. Later studies showed that *M. libycus* is the plague reservoir in this focus and the primary vectors are *X. conformis* and *C. laeviceps* fleas (26, 27).

The Transcaucasian desert plague foci are spread across approximately 500,000 ha, with each focus covering 40,000–200,000 ha. The foci are in a semi-desert zone near the Iori and Alazani rivers. Epizootics are severe in this area and *Y. pestis* strains that are isolated tend to be highly virulent; most of these strains were isolated from dead rodents. The epizootic process is spread across several individual foci with the same geographic features.

Georgian Anti-Plague Stations have conducted active surveillance in these foci since the early 1900s and have collected over 120 *Y. pestis* strains from these regions (28). The National Center for Disease Control and Public Health (NCDCPH) collection currently contains 46 strains, 40 of which were isolated from Georgian plague foci between 1966 and 1997 (25).

The differences between *Y. pestis* strains, including Georgian strains, on a genomic level have been extensively studied (25, 29–32). It is likely that some of these strain variations are also reflected at the proteomic level as the final products of gene expression. At the time of publication, no comparative studies of trans-Caucasian *Y. pestis* strains at the proteomic level were available in published literature.

A neighbor-joining tree was generated using multilocus variable number tandem repeat analysis for the 46 unique *Y. pestis* strains in the NCDCPH collection in Tbilisi, Georgia (30). Four strains of *Y. pestis* located at different positions on this tree were selected for comparative proteomic analysis.

Early studies of *Yersinia* physiology uncovered the low calcium response, whereby bacterial cultures grown in rich medium at an elevated temperature (37°C) exhibit a growth defect upon chelation of calcium ions. This defect was shown to be a result of a type III secretion system in *Y. pestis*, the Ysc TTSS, and is responsible for the secretion of virulence factors known as *Yersinia* outer proteins (YOPS) (33). This secretion system can be activated *in vitro* and virulence factors can be released into the medium when *Y. pestis* is grown at 37°C in the presence of millimolar concentrations of calcium (34). The *Y. pestis* proteome was previously examined using two-dimensional (2-D) gel electrophoresis (35, 36). These studies showed that virulence factors were induced at 26 or 37°C in the presence of 2.5 mM Ca^{2+} (a concentration similar to that in mammalian plasma). The *Y. pestis* proteome varies as a function of temperature and calcium, and expression of virulence factors clearly depends on these physiological conditions (37); thus, differences between the proteomes of *Y. pestis* strains could be masked under one set of physiological conditions, but expressed under another.

In this study, we examined and characterized proteomes of *Y. pestis* strains from the trans-Caucasian area as a function of temperature and calcium, which were used to affect induction of virulence. *Y. pestis* strains were grown at different

temperatures in the presence and absence of calcium ions (Ca^{2+}) and their proteomes were compared by 2-D gel electrophoresis and mass spectrometry (MS) and confirmed by Western blotting.

MATERIALS AND METHODS

Yersinia pestis Strains

The following strains of *Y. pestis* were chosen for comparative proteomic studies: 1390, 1853, 8787, and 2944. These strains were provided by the NCDCPH in Tbilisi, Georgia. Strains 1390, 1853, and 8787 were isolated in Ninotsminda, Georgia, in 1979, 1980, and 1992, respectively; strain 2944 was isolated in Kabardino-Balkaria, Russia, in 1975.

Yersinia pestis Growth with Temperature and Calcium Concentration Changes

The following procedure was carried out in quadruplicate for each *Y. pestis* strain included in the study. Cultures were grown overnight in Mueller-Hilton broth at 28°C with continuous shaking. After incubation, 0.1 mL of each culture was transferred into 15 mL of fresh broth. Two aliquots were incubated at 37°C, one of which was adjusted with 0.4 M $CaCl_2$ (15 μL) to a final concentration of 4 mM; 15 μL of sterile distilled water was added to the second aliquot. The remaining two aliquots were treated as described above but incubated at 28°C. All aliquots were incubated for another 4 h at the specified temperatures. Cells were harvested during the exponential phase of growth. Aliquots were centrifuged at 3,000 × *g* for 10 min, and duplicate bacterial pellets from strains under each set of physiological conditions were prepared for one-dimensional (1-D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-D gel electrophoresis.

Preparation for 1-D SDS-PAGE

Bacterial pellets were resuspended in 5% SDS and incubated at 95°C for 10 min. Lysates were centrifuged at 15,000 × *g* for 15 min, and the supernatant was collected and tested for sterility; inactivated samples were inoculated and grown in tryptic soy broth with 0.0125% phenol red for 72 h at 28°C. After incubation, samples were assessed for color change or turbidity; samples were considered sterile if neither color change nor turbidity were observed.

Preparation for 2-D Gel Electrophoresis

Bacterial pellets were resuspended in buffer (25 mM Tris-acetate, pH 7.8, 5 mM EDTA, 150 μg/mL lysozyme, 2 mM PMSF, 0.05% Triton X-100, and 1 mM benzamidine). Samples were incubated for 30 min at 20°C with intermittent vortexing. Lysates were centrifuged at 20,000 × *g* for 60 min at 4°C. The supernatant was collected and 20% CHAPS stock solution was added to a final concentration of 2% (w/v), followed by incubation at 95°C for 10 min. After incubation, the supernatant was tested for sterility as previously described.

Protein Quantification

A micro-BCA kit (Pierce, Thermo Scientific) was used to quantify the protein concentration in supernatants. Protein quantification was performed in quadruplicate for each sample with the appropriate buffer controls, according to the manufacturer's instructions.

2-D Gel Electrophoresis

Sample Preparation and Isoelectrofocusing

Isoelectrofocusing (IEF) strips (linear pH 3–10) were rehydrated in 8M urea, 0.5% Triton X-100, 0.5% Pharmalyte 3–10, and 30 mM DTT overnight. Protein samples (30 μg) were loaded onto rehydrated strips in buffer containing 7M urea, 2M thiourea, 2% CHAPS, 2% Triton X-100, 0.1% ASB-14, 2-mercaptoethanol, 2% Pharmalyte 3–10, and bromophenol blue. IEF was carried out at 500 V for 3 h and 3,500 V for 18 h ± 30 min.

Equilibration

Strips were equilibrated for 15 min in a buffer containing 0.05M Tris-HCl (pH 6.8), 6M urea, 30% glycerol, 3% SDS, and 1% DTT, followed by equilibration in the same buffer with 2.5% DTT iodoacetamide instead of 1% DTT for 15 min.

SDS Electrophoresis

SDS electrophoresis was run on a 1-mm thick, 12.5% polyacrylamide gel at 25°C, first at 10 mA per gel at 80 V for 1 h, and then at 12 mA per gel at 150 V for 17 h.

Staining, Scanning, and Analysis

The gels were stained with a silver stain kit (GE-Healthcare), omitting the glutaraldehyde step. Silver-stained gels were scanned with an image scanner (Labscan 6.0). Images were digitalized and processed using Image Master 2D platinum 7.0.

In each series of experiments, the supernatants of the four *Y. pestis* strains were analyzed concurrently for each physiological condition. Proteins that exhibited at least 1.5-fold difference between the strains were selected. The relative intensities of protein spots coinciding by location (isoelectric point and molecular weight) from different experiments were compared by *t*-test. The significantly differentially expressed protein spots ($p < 0.05$) were excised, destained, and stored at −20°C until MS analysis.

In-Gel Digestion and MS Analysis

Excised proteins were reduced with TCEP and alkylated with iodoacetamide. Samples were then treated with acetonitrile, dried, and rehydrated in activated trypsin (Thermo Scientific, Pierce) to begin digestion; proteins were digested at 37°C for 4 h. Digested samples were processed using nanospray ionization tandem HPLC-MS/MS CID performed with helium (Finnigan LTQ, Thermo Scientific). MS/MS spectra data was analyzed using SEQUEST (Proteome Discoverer 2.0), searching against UniProt UniRef100 databases.

Antibodies

Antibodies against F1 antigen were obtained from Life Science-Meridian (cat. # C86308M). Polyclonal antibodies against

DNA-binding protein H-NS, tellurium-resistance protein, and outer membrane protein C, porin, were raised in rabbits against the following peptide sequences:

- H-NS: EMLEKLEVVVN (amino acids 28–38)
- Tellurium-resistance protein: PADVDKIVFVVT (amino acids 99–110)
- Outer membrane protein C, porin: NTDDIVAVGMVYQ (amino acids 332–344)

Antibodies against H-NS and tellurium-resistance protein were affinity-purified on corresponding peptide columns; antibodies against outer membrane protein C, porin were purified on protein-A Sepharose columns. The specificities of antibodies were tested by inhibition of binding with immunizing peptides using Western blotting.

1-D SDS-PAGE and Western Blotting

Laemli SDS sample buffer was added to protein aliquots (30 μg). Samples were then analyzed by SDS-PAGE, followed by Western blotting. After electrophoretic transfer onto nitrocellulose membranes, protein bands were detected by staining with Ponceau S to confirm the uniform loading of the gel. Nitrocellulose membranes were sectioned according to the molecular weight standard and were then probed with primary antibodies against the following antigens: F1; tellurium-resistance protein; outer membrane protein C, porin; and DNA-binding protein H-NS. Because F1 and outer membrane protein C, porins are of a similar molecular weight, separate electrophoresis were carried out for each antigen.

Standard immunochemical procedures were carried out, and bands were visualized using peroxidase-labeled secondary antibodies (anti-rabbit) and a SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pierce). Intensifying screens were used to expose the blots to X-ray films pre-flashed with Sensitize (Amersham). The densities of the protein bands were measured using LabWorks 4.0 software (UVP).

The autoradiographs were calibrated using combined protein from the four strains (15, 30, 45, and 60 μg total protein). Standard curves were prepared for each protein by plotting the optical density of the immunostained band against the protein concentration. The least squares regression showed a significant fit to a straight line for all standards. In experimental samples, to calculate protein amount, the optical density of each band was divided by the optical density of the 30-μg total protein standard; this value was considered to be the "relative amount" of protein (e.g., F1, Tellurium-resistance protein, and H-NS) (38).

In vitro Cytotoxicity Assay

In vitro cytotoxicity of *Y. pestis* strains and the vaccine strain EV was evaluated based on strain ability to induce apoptosis in macrophage cultures. Apoptosis was assayed using a colorimetric caspase-3 assay kit (Sigma-Aldrich, cat. #CASP-3-C) according to the manufacturer's instructions. This kit measures the activity of caspase-3, one of the critical enzymes of apoptosis, and includes a specific inhibitor for precise measurement of caspase-3 activity.

Macrophages were cultivated as previously described (39, 40). Briefly, J774A.1 mouse macrophages were grown in DMEM supplemented with 10% heat-inactivated FBS. Cultures were incubated for 72 h at 37°C in a 5% CO_2 atmosphere, after which the wells were washed to remove non-adherent cells.

To each well, 100 μL of *Y. pestis* (3×10^8 cfu/mL) was added and incubated at 37°C for 3 h. Plates were centrifuged at $600 \times g$ for 5 min at 4°C, washed once with 1 mL of PBS per well, and 1× lysis buffer was added (100 μL/10^5cells). After 15 min of incubation on ice, the lysate was centrifuged at $3{,}000 \times g$ for 5 minutes at 4°C, and the supernatant was stored at −70°C. Supernatants were filtered, divided into aliquots, and tested for sterility as previously described. Cell lysates and positive controls were brought to the required volume with 1× assay buffer and incubated for 2 h at 37°C with caspase-3 substrate. For each aliquot, caspase-3 inhibitor was included in addition to the peptide substrate, and parallel measurements were taken. The amount of *p*-nitroaniline (pNA) released in the assay was measured using a spectrophotometer ($OD_{405\,nm}$) and the concentration was determined by the standard curve. These values were subtracted from the values obtained without the inhibitor. The protein amount was determined in cell lysates, and enzyme activity expressed as nanomoles of pNA released per minute per 1 mg of cell lysate protein.

Statistical Analysis

The relative amounts of proteins were subjected to analysis of variance (ANOVA) by strain (1390, 1853, 2944, and 8787) and physiological condition (28°C, 28°C with Ca^{2+}, 37°C, 37°C with Ca^{2+}). Results from different strains grown under the same physiological conditions were compared by *t*-test.

Caspase-3 activity data was analyzed by strain using one-way ANOVA. Planned comparisons were done by *t*-test. All *t*-tests were two-tailed unless otherwise indicated.

RESULTS

2-D Gel Electrophoresis

The 2-D gel electrophoresis of *Y. pestis* protein extracts was carried out on IEF strips with two pH gradients: 3.0–10.0 and 3.0–5.6 (**Figures 1A,B**). The majority of the proteins on the 3.0–10.0 pH gradient gels were concentrated between pH 3.0 and 6.0 (**Figure 1A**), so 2-D gel electrophoresis was repeated using strips with a pH gradient from 3.0 to 5.6 for better resolution of protein bands (**Figure 1B**).

The comparison of images of different strains revealed consistent differential expression of bands. The most significant differences were observed at 28°C. Strains were divided into two groups according to their differences: (1) 1853 and 1390 and (2) 2944 and 8787. Differential expression of certain proteins was significantly higher in the first group of bacterial strains. MS analysis was used to determine the identity of the excised proteins (**Table 1**). To validate the significant differences between the proteins, quantitative Western blotting was used to study four of the five identified proteins. The criteria for the selection of proteins that we studied were the following reasons: (i) availability of the commercial antibodies, (ii) our ability to produce custom antibodies, and (iii) differential expression profiles of these proteins under the various growth conditions.

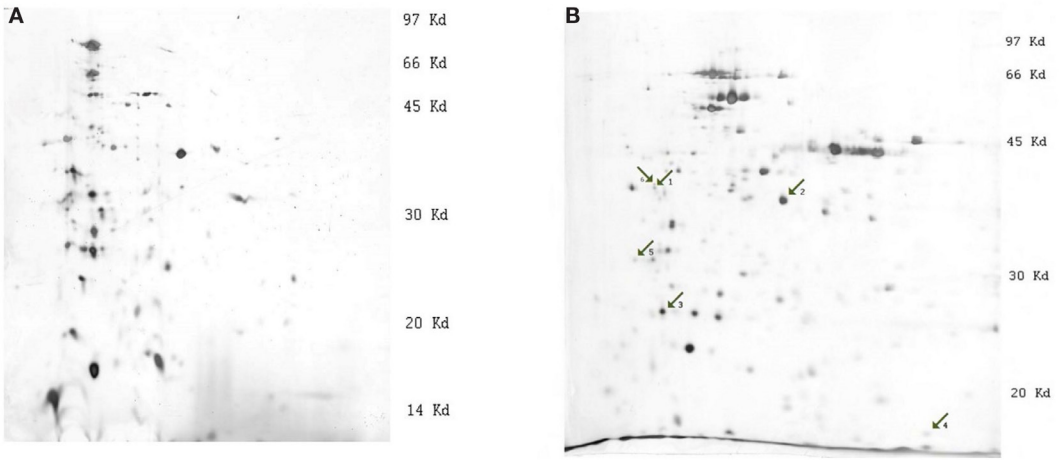

FIGURE 1 | Images of silver stained 2-D gels of *Y. pestis* protein extracts (strain 1853, condition 28°C). **(A)** 2-D electrophoresis on strips with pH linear gradient 3.0–10.0, **(B)** 2-D electrophoresis on strips with pH linear gradient 3.0–5.6. The numbered arrows indicate the positions of differentially expressed proteins: 1, outer membrane protein C, porin; 2, outer membrane protein C2 porin; 3, Tellurium-resistance protein; 4, DNA-binding protein H-NS; and 5, F1 capsule antigen.

TABLE 1 | Differentially expressed proteins identified by 2-D electrophoresis by condition and strain.

Protein	Condition/strain
Outer membrane protein C, porin	28°C, 1853 and 1390 > 2944, 8787
Tellurium-resistance protein	28°C, 1853 and 1390 > 2944, 8787
DNA-binding protein H-NS	28°C, 1853 and 1390 > 2944, 8787
F1 antigen	37°C, 37°C + Ca^{2+} and 28°C, 1853 and 1390 > 2944, 8787
Outer membrane protein-C2, porin	28°C, 1853 and 1390 > 2944, 8787

Immunostaining

Standards were prepared as described above (15, 30, 45, and 60 μg total protein). For these standards, the optical densities of the bands immunostained for F1, outer membrane protein C, porin, tellurium-resistance protein, and DNA-binding protein H-NS were linearly related to the amounts of proteins in the bands (**Figures 2A–D**).

Antibodies against F1 reacted with a protein band of molecular weight of approximately 36–38 kDa (**Figure 2A**). The molecular weight of F1 antigen is 17.7 kDa, but it migrates as a dimer in SDS electrophoresis (41). Outer membrane protein C, porin antibodies reacted with a protein band of apparent molecular weight approximately 38 kDa, which corresponds to the expected size of the target protein (**Figure 2B**). Tellurium-resistance protein antibodies reacted with a protein band of 20 kDa (**Figure 2C**) and DNA-binding protein H-NS antibodies reacted with a protein of apparent molecular weight 15–16 kDa (**Figure 2D**). Both of these weights corresponded to the expected size of the target proteins.

F1 Antigen

Y. pestis strain and growth condition were both found to be significant factors in the expression of F1 when analyzed by two-way ANOVA (respectively $F_{3,63} = 5.40$, $p = 0.002$; $F_{3,63} = 9.95$, $p = 0.0001$). As expected from 2-D electrophoresis data, the most significant changes were observed at 37°C (**Figure 3C**). The mean amount of F1 antigen was significantly higher in strain 1390 than in strains 2944 ($t = 2.65$, $p = 0.038$) and 8787 ($t = 2.85$, $p = 0.029$). The mean amount of F1 was also higher in strain 1390 than strain 8787 at 28°C ($t = 3.47$, $p = 0.013$; **Figure 3A**). At 28°C with Ca^{2+}, and 37°C with Ca^{2+}, mean amounts of F1 antigen in 1390 and 1853 were not significantly higher than mean amounts in 8787 and 2944 (**Figures 3B,D**, respectively).

Outer Membrane Protein C, Porin

Two-way ANOVA revealed significant differences in expression of outer membrane protein C, porin, between strains ($F_{3,63} = 8.53$, $p = 0.0001$). The largest significant differences were observed at 28°C (**Figure 4A**). The mean amount of outer membrane protein C, porin was significantly higher in strain 1853 than strains 2944 ($p = 0.02$) and 8787 ($p = 0.002$), and in strain 1390 than strain 8787 ($p = 0.041$). The amount of outer membrane protein C, porin was significantly higher in strain 1853 than 8787 at 28°C with Ca^{2+} ($p = 0.025$, **Figure 4B**). Statistically significant differences were not observed between strains grown under other conditions (**Figures 4C,D**).

Tellurium-Resistance Protein

For the tellurium-resistance protein, the effects of strain and growth conditions on expression (respectively $F_{3,63} = 5.49$, $p = 0.002$; $F_{3,63} = 21.03$, $p = 0.0001$) were significant by two-way ANOVA. The mean amount of protein at 28°C was significantly higher in strain 1390 than strains 2944 ($p = 0.019$) and 8787 ($p = 0.001$), and the amount in strain 1390 was significantly higher than in strain 8787 ($p = 0.006$). At 28°C with Ca^{2+}, the mean value of protein in 1853 was significantly higher than 2944 ($p = 0.044$) (**Figures 5A–D**); no other significant differences were observed.

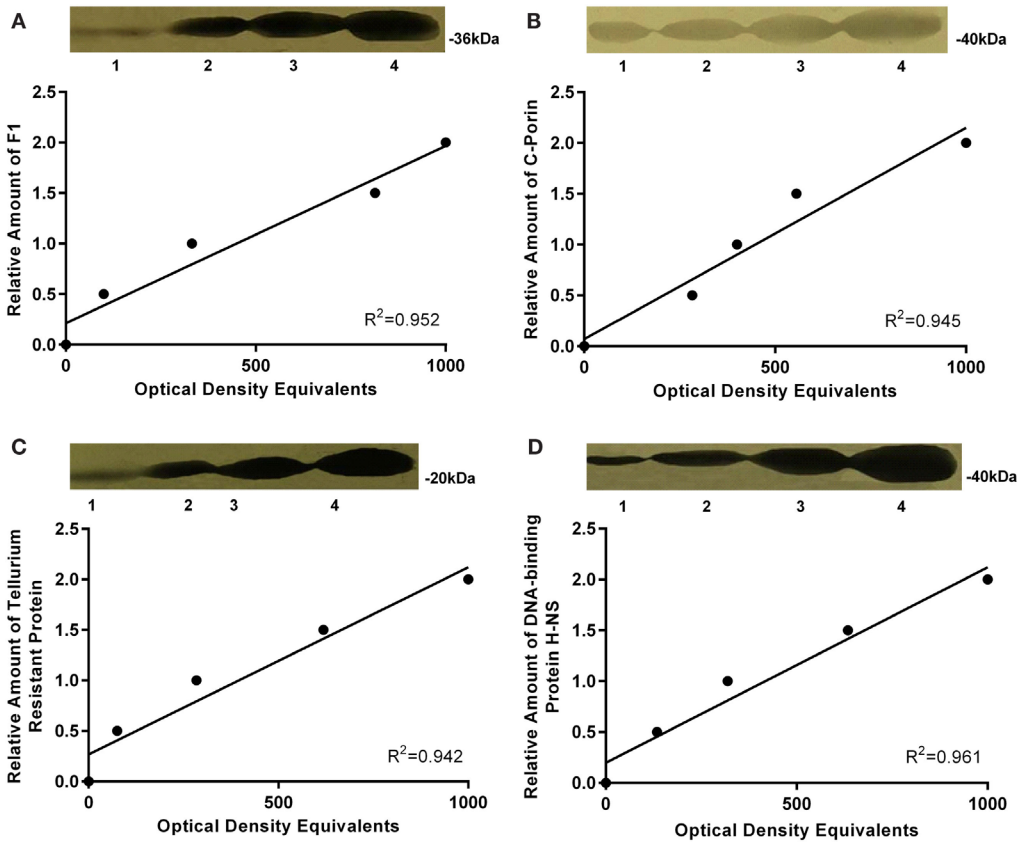

FIGURE 2 | Western blot autoradiographs and calibration plots. Western blot autoradiographs for single standard sample of the mixture of all *Y. pestis* strains protein extracts containing 15, 30, 45, and 60 μg of protein, respectively. Calibration plots are shown below each autoradiograph and reflect the linear response of immunostaining with the corresponding amount of loaded protein for **(A)** F1 antigen, **(B)** outer membrane protein C, porin, **(C)** Tellurium, and **(D)** DNA-binding protein H-NS.

DNA-Binding Protein H-NS

None of the differences in expression of DNA-binding protein H-NS between strains or under different growth conditions were significant by ANOVA (**Figures 6A–D**).

In vitro Cytotoxicity Assay

Caspase-3 activity of *Y. pestis* strains was compared using one-way ANOVA; activity was significantly different between strains ($F_{4,\ 19} = 22.15$, $p = 0.0001$; **Figure 7**). The highest activity of caspase-3 was observed in strain 1853, which significantly exceeded the activity in strains 1390 ($t = 3.24$, $p = 0.018$), 8787 ($t = 9.35$, $p = 0.0001$), and EV ($t = 11.20$, $p < 0.0001$). Caspase-3 activity in strain 2944 was significantly higher than in strains 8787 and EV ($t = 5.69$, $p = 0.001$). Caspase-3 activity was also significantly higher in strain 1390 than strains 8787 ($t = 3.55$, $p = 0.012$) and EV ($t = 4.48$, $p = 0.004$) (**Figure 7**). No other significant differences were observed (by two-tailed *t*-test).

DISCUSSION

Genetic polymorphisms are often associated with various *Y. pestis* strains isolated from natural foci in the Republic of Georgia, but it is not known if these polymorphisms affect gene expression or if there are differences between the proteomes of various strains (42, 43). The results of this study demonstrate that strains of *Y. pestis* isolated from natural foci in the Republic of Georgia differ at the proteomic level as well as the genetic level.

The main goal of this research was to identify a set of candidate proteins that are differentially expressed across the different Georgian *Y. pestis* isolates. A combination of 2-D electrophoresis and MS was used to identify several candidate proteins. However, 2-D electrophoresis with silver staining is not a strong quantitative approach, even when analyzed with even the most sophisticated software; therefore, further validation was required. To this end, we used quantitative Western blotting to measure the levels of proteins (the final products of gene expression). From candidate proteins, the following proteins were chosen as the focus of the experiments: F1 antigen; tellurium-resistance protein; outer membrane protein C, porin, and DNA-binding protein H-NS.

F1 antigen is widely accepted as a virulence factor of Y. pestis. F1 is encoded by the caf1 gene located on the large 100-kb pFra plasmid, which is unique to *Y. pestis*. F1 antigen is synthesized as a 15–16 kDa monomer and forms a large homopolymer

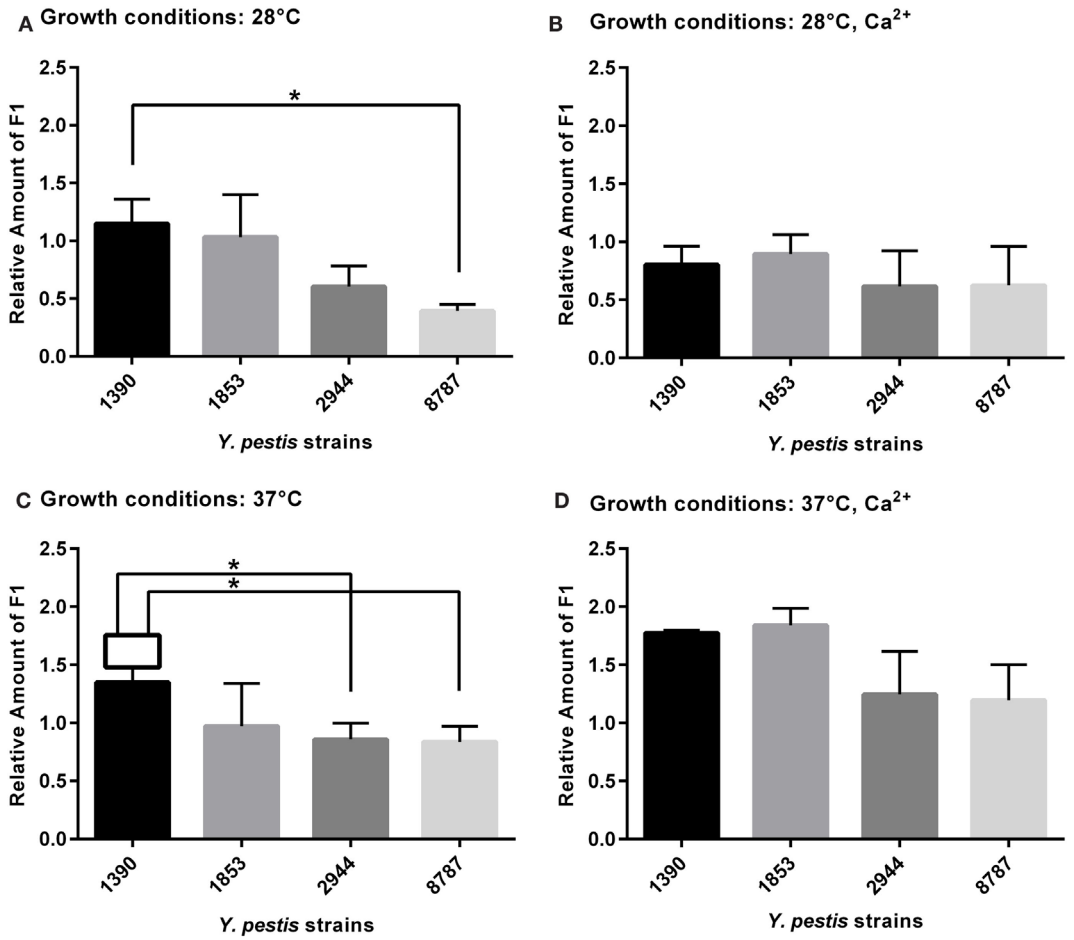

FIGURE 3 | Relative amount of F1 antigen expressed by *Y. pestis* strains 1390, 1853, 2944, and 8787 at physiological growth conditions (A) 28°C; (B) 28°C, Ca^{2+}; (C) 37°C; and (D) 37°C, Ca^{2+}. The relative amount of protein expressed was calculated from immunostained bands, by dividing the optical density of the band by the optical density of the corresponding 30 μg total protein standard. Data represents the mean + SEM. *Indicates significant differences ($p < 0.05$).

(>200 kDa) on the bacterial cell surface in a stacked ring structure composed of heptamers (44, 45). The secretion and assembly of F1 require the *caf1M* and *caf1A* genes, which are homologous to the chaperone and usher protein families required for biogenesis of pili. F1 may be involved in the ability of *Y. pestis* to prevent uptake by macrophages, thus protecting *Y. pestis* from the host's innate immune response (45). F1-antigen is also known to inhibit bacterial adhesion to epithelial cells (45).

As a differentially expressed protein, F1 antigen was detected on gels with bacterial extracts grown at 37°C and at 37°C with Ca^{2+}. Western blotting analysis verified that the mean amount of F1 antigen was significantly higher in strain 1390 than in strain 2944 and 8787 at 37°C. The mean amounts of F1 antigen expressed at 37°C with Ca^{2+} were not significantly higher in strains 1390 and 1853 than strains 2944 and 8787. These results indicate that expression of F1 antigen differs from strain to strain. Further, the F1 antigen has been well-documented to be temperature regulated. Prior work demonstrates enhanced F1 expression at >35°C. Considering that our findings revealed enhanced expression at 37°C relative to 28°C (most evident when grown in the presence of calcium), this provides additional authenticity to our results (46).

Tellurium is a trace element that belongs to the same chemical group as selenium, sulfur, and oxygen. Tellurite oxyanions are highly toxic for most forms of life even at micromolar levels (47). Toxicity of tellurium may be mediated partly by reactive oxygen species that are generated as by-products of tellurite reduction (48). The primary functions of tellurium-resistance proteins are not known, although some sources suggest that they are involved in detoxification of antimicrobial compounds produced by host macrophages (47). *Y. pestis* cells in natural rodent hosts multiply initially in macrophage phagolysosomes. Survival and multiplication of *Y. pestis* in this new environment likely requires compensatory mechanisms involving expression of specific proteins compared to those expressed during extracellular growth. Indeed, 2-D electrophoresis and MS analysis has shown that intracellular and extracellular *Y. pestis* proteomes differ from each other in the expression of 12 proteins (49). Differentially expressed proteins that were upregulated inside the macrophages include tellurium-resistance proteins and

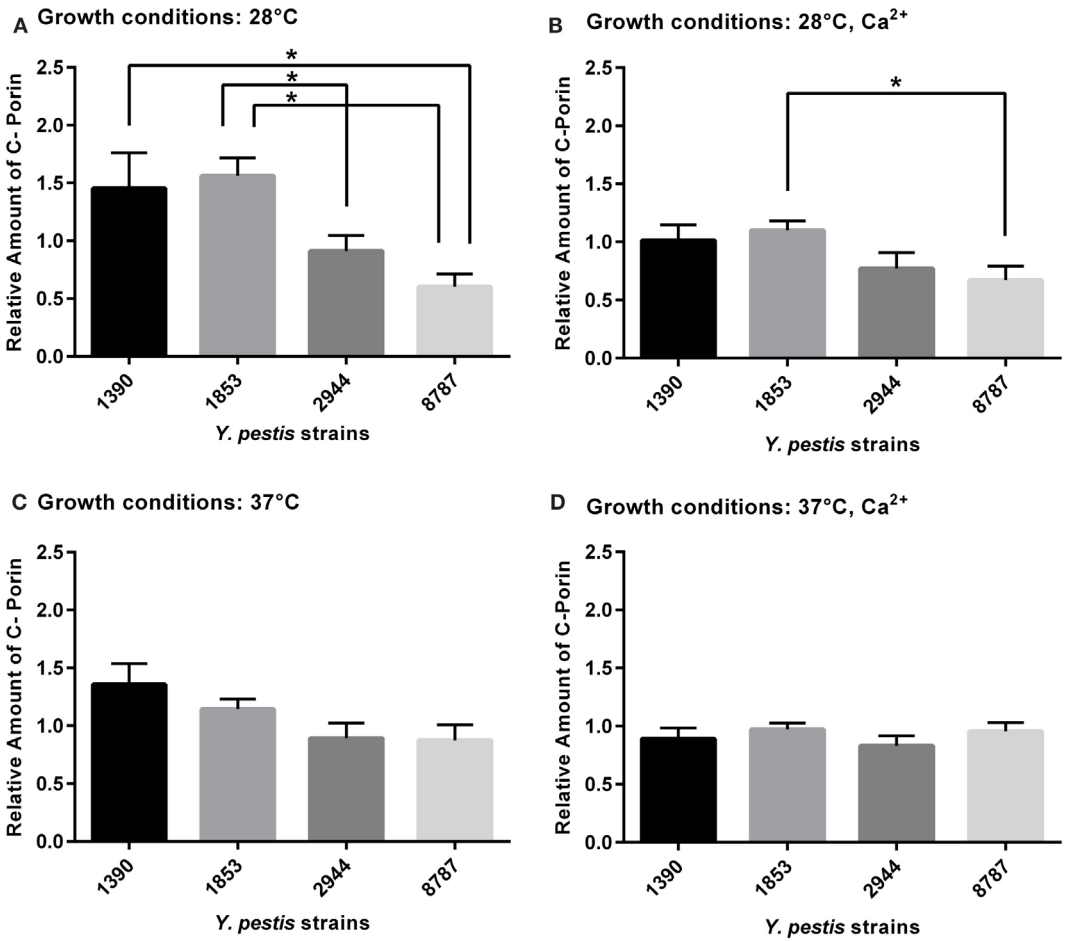

FIGURE 4 | Relative amount of outer membrane protein C, porin expressed by *Y. pestis* strains 1390, 1853, 2944, and 8787 at physiological growth conditions (A) 28°C; (B) 28°C, Ca^{2+}; (C) 37°C; and (D) 37°C, Ca^{2+}. The relative amount of protein expressed was calculated from immunostained bands, by dividing the optical density of the band by the optical density of the corresponding 30 μg total protein standard. Data represent the mean + SEM. *Indicates significant differences ($p < 0.05$).

also DNA-binding protein H-NS (49). Since the survival and multiplication of *Y. pestis* inside macrophages may be enhanced tellurium-resistance proteins, the strain-specific variations in tellurium-resistance protein expression might influence virulence during mammalian infection. The results of these experiments suggest that strains 1390 and 1853 are expressing tellurium-resistance protein at higher levels than strains 2944 and 8787.

Iron is an essential element for the survival of many microorganisms and its acquisition by bacteria can be central to the outcome of an infection (45). Recent data indicate that outer membrane proteins, including protein C, are transferrin binding proteins and could be involved in the acquisition of iron for growth within their host (47). Differences in the expression of outer membrane protein C, porin, between the strains were generally analogous to differences in expression of tellurium-resistance protein. Significant differences between the strains were observed at 28°C; the mean amount of protein was higher in strains 1390 and 1853 than strains 2944 and 8787.

DNA-binding protein H-NS belongs to a group of nucleoid-associated proteins, which are associated with the chromosome. They possess substantial non-specific DNA-binding affinity and have two major functions: gene regulation and chromosome organization (50). As mentioned above, this protein in is upregulated in *Y. pestis* inside of macrophages (49). DNA-binding protein H-NS was identified as a significantly differentially expressed protein when analyzed on silver-stained 2-D gels, but further quantitative analysis with Western blotting indicated that differences in expression between strains were not significant. The greatest difference, which was observed between strains 1390 and 8787, was only significant in a one-tailed *t*-test.

It is well documented that cell death plays a central role in host-pathogen interactions by eliminating the pathogen's replicative niche and/or by eliminating immune cells and evading antimicrobial effector mechanisms (51). In these experiments, the ability to induce apoptosis in macrophage cell cultures was used to determine the toxicity of *Y. pestis* isolates. Macrophages

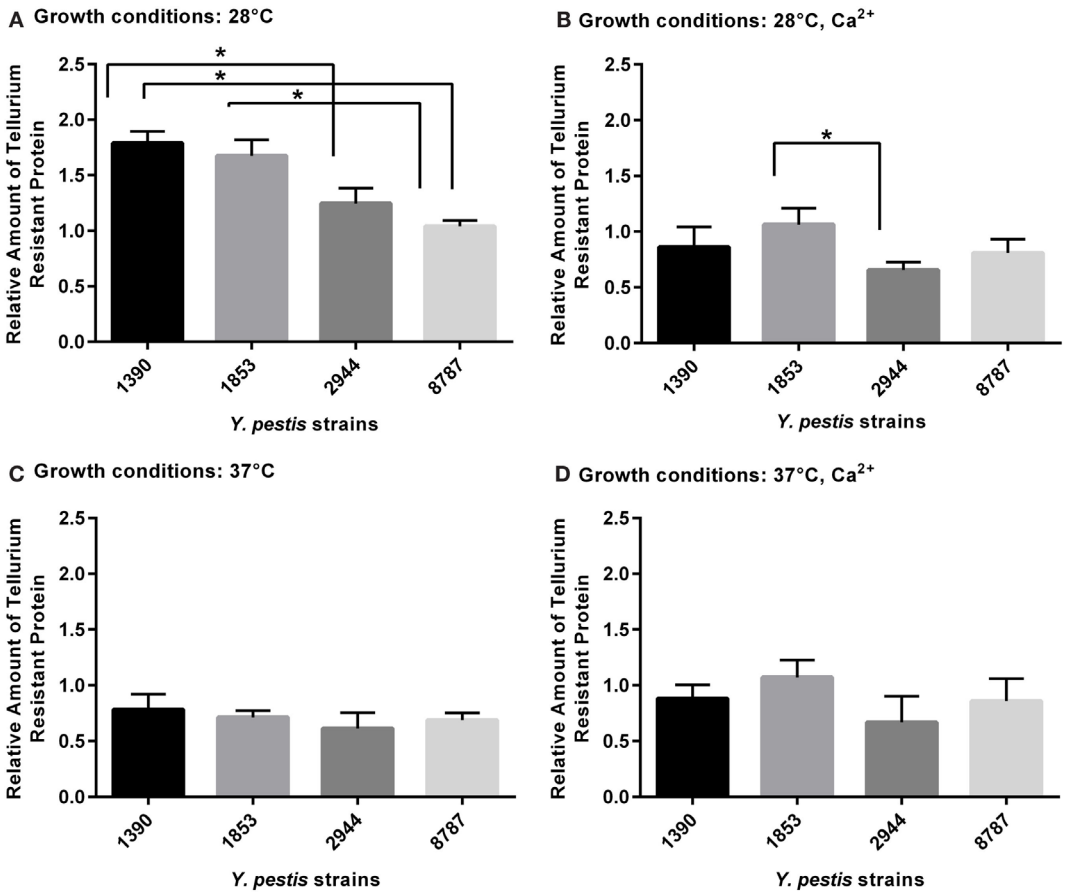

FIGURE 5 | Relative amount of tellurium-resistance protein expressed by *Y. pestis* strains 1390, 1853, 2944, and 8787 at physiological growth conditions (A) 28°C; (B) 28°C, Ca^{2+}; (C) 37°C; and (D) 37°C, Ca^{2+}. The relative amount of protein expressed was calculated from immunostained bands, by dividing the optical density of the band by the optical density of the corresponding 30 μg total protein standard. Data represent the mean + SEM. *Indicates significant differences ($p < 0.05$).

are responsible for detecting, engulfing, and destroying pathogens and their apoptosis terminates the host's immune response. Caspase-3 is a mediator of the pathogenic effect of *Y. pestis* in the livers of C57BL/6 mice (52); therefore the apoptosis activity, as indicated by caspase-3 expression, could be a measure of the virulence of *Y. pestis* isolates. Caspase-3 activity differed significantly among the study strains: the lowest significant expression was observed in strains EV and 8787, and the highest was observed in 1853. The apoptosis-inducing activities in strains 2944 and 1390 were also significantly higher than in strains 8787 and EV.

The overall comparative analysis of studied strains indicates that strain 8787 is distinct from other strains due to a low expression of virulence factors and cytotoxic activities. According to the neighbor-joining tree generated for 46 *Y. pestis* strains from the NCDCPH, strain 8787 is different from strains 1390, 1853, and 2944. Strains 1853 and 1390 are in close proximity to each other. The levels of expression of F1, tellurium-resistance protein, and outer membrane protein C, porin suggest that strains 1853 and 1390 are close to each other. According to the cytotoxicity assay, strains 1853 and 1390 are significantly different from each other and are both different from 8787 and 2944. Strain 2944 is characterized by a lower expression of the studied proteins and high cytotoxic activity, which is likely driven by factors other than those studied. Thus, the results obtained confirm both relatedness and differences between the strains.

Plasminogen activator, Pla is encoded by the pPCP plasmid and is one of the major virulence factors in *Y. pestis* (53). According to one study, the pPCP plasmid is present in all strains of *Y. pestis* from the NCDCPH collection (25), but in another series of experiments, the plasmid was found only in three strains (2944, 2614, and 790) (25, 30). If the plasmid is only found in three strains, the high cytotoxic activity of strain 2944 could be explained by the presence of the pPCP plasmid. According to the blotting data, the Pla is expressed in all of these strains (data not shown). We also consider the possibility that Pla gene from pPCP plasmid was integrated in to the bacterial chromosome, which has been reported in previous studies.

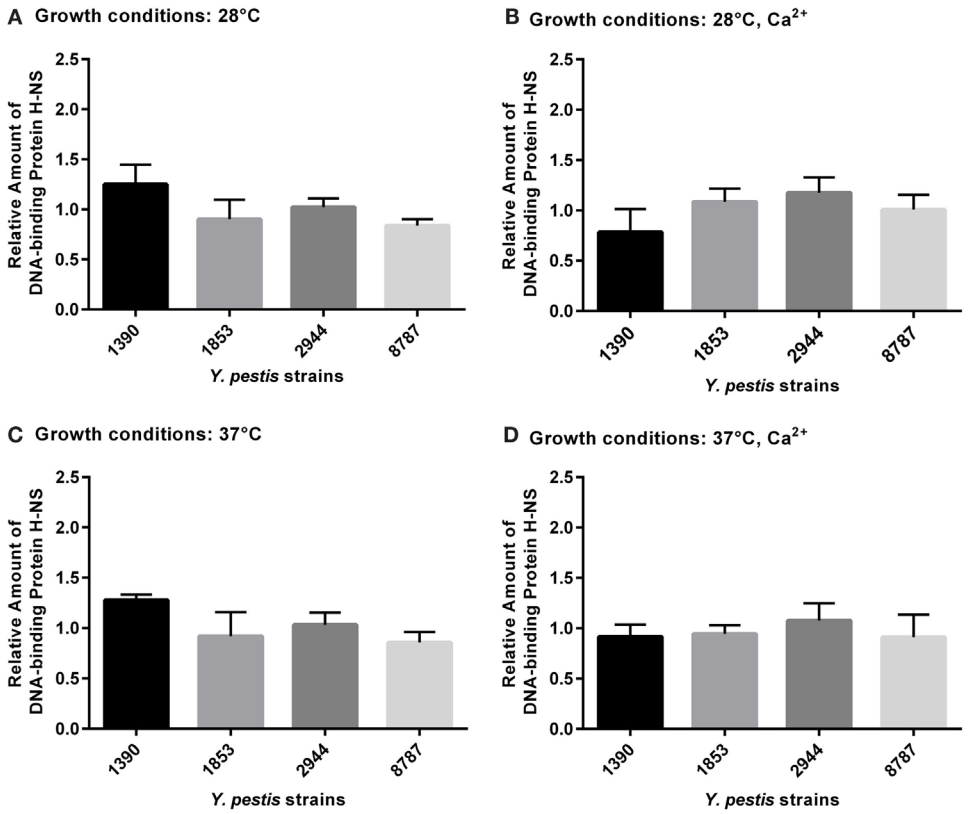

FIGURE 6 | Relative amount of DNA-binding protein H-NS expressed by *Y. pestis* strains 1390, 1853, 2944, and 8787 at physiological growth conditions (A) 28°C; (B) 28°C, Ca^{2+}; (C) 37°C; and (D) 37°C, Ca^{2+}. The relative amount of protein expressed was calculated from immunostained bands by dividing the optical density of the band by the optical density of the corresponding 30 μg total protein standard. Data represent the mean + SEM. *Indicates significant differences ($p < 0.05$).

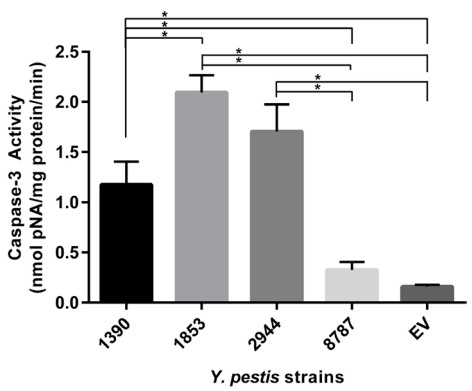

FIGURE 7 | Macrophage caspase-3 activity under the influence of *Y. pestis* strains 1390, 1853, 2944, 8787, and EV. *In vitro* cytotoxicity of *Y. pestis* strains and the vaccine strain EV was evaluated based on their ability to induce apoptosis in macrophage cultures. Data represent the mean + SEM. *Indicates significant differences ($p < 0.05$).

The proteome of *Y. pestis* as a function of changes in temperature and calcium provides information regarding the expression levels of virulence-associated factors, putative virulence factors, and metabolic and housekeeping proteins, as well as potential novel virulence determinants. Future studies using altered gel formulations to examine lower-molecular-weight proteins, and experiments to control the proteolytic activity of Pla will provide more information about these *Y. pestis* strains. Although, we can infer that there are differences between strains and the functions of the identified differentially expressed proteins, future studies using mutant strains of *Y. pestis* are needed. The proteomic analysis of these strains reported here allows for future comparisons of clinical and environmental isolates across the Caucasus.

ACKNOWLEDGMENTS

This research was made possible by financial support provided by the U.S. Department of Defense (DoD), Defense Threat Reduction Agency (DTRA), through the Biological Threat Reduction Program's (BTRP) TADR Activity Project TAP-6, in Georgia. The findings, opinions, and views expressed herein belong to the authors and do not reflect an official position of any organization listed nor does it reflect the official policy or position of the Department of the Navy, DoD, nor the U.S. Government. The authors thank Attimo Research and Development and Sonya Narodny for editorial assistance.

REFERENCES

1. Anisimov AP, Lindler LE, Pier GB. Intraspecific diversity of *Yersinia pestis*. *Clin Microbiol Rev* (2004) **17**(2):434–64. doi:10.1128/CMR.17.3.695.2004
2. Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Plague as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *JAMA* (2000) **283**(17):2281–90. doi:10.1001/jama.283.17.2281
3. Cui Y, Yang X, Xiao X, Anisimov AP, Li D, Yan Y, et al. Genetic variations of live attenuated plague vaccine strains (*Yersinia pestis* EV76 lineage) during laboratory passages in different countries. *Infect Genet Evol* (2014) **26**:172–9. doi:10.1016/j.meegid.2014.05.023
4. Hinnebusch BJ. The evolution of flea-borne transmission in *Yersinia pestis*. *Curr Issues Mol Biol* (2005) **7**(2):197–212.
5. Perry RD, Fetherston JD. *Yersinia pestis* – etiologic agent of plague. *Clin Microbiol Rev* (1997) **10**(1):35–66.
6. Wimsatt J, Biggins DE. A review of plague persistence with special emphasis on fleas. *J Vector Borne Dis* (2009) **46**(2):85–99.
7. Acha PN, Szyfres B. *Zoonoses and Communicable Diseases Common to Man and Animals*. Washington, DC: Pan American Health Organization (2001). p. 218–26.
8. Bacon M, Drake CH. Comparative susceptibility of various species of mice native to Washington to inoculation with virulent strains of *Pasteurella pestis*. *J Infect Dis* (1958) **102**(1):14–22. doi:10.1093/infdis/102.1.14
9. Baltazard M, Karimi Y, Eftekhari M, Chamsa H, Mollaret HH. *Interepizootic Conservation of Plague in its Inveterate Foci: Working Hypotheses*. Frederick, MD: Army Biological Labs (1964).
10. Hubbert WT, Goldenberg MI. Natural resistance to plague: genetic basis in the vole (*Microtus californicus*). *Am J Trop Med Hyg* (1970) **19**(6):1015–9.
11. Azizi MH, Azizi F. A history of the human plague in Iran. *Arch Iran Med* (2010) **13**(6):563–9. doi:010136/AIM.0022
12. Biggins DE, Kosoy MY. Influences of introduced plague on North American mammals: implications from ecology of plague in Asia. *J Mammal* (2001) **82**(4):906–16. doi:10.1644/1545-1542(2001)082<0906:IOIPON>2.0.CO;2
13. Mostafavi E, Asadmanesh K, Nadaf S, Hanifi H, Hanifi M, Behzadi-yousefi M. Study of the plague infection in rodents and carnivores in Western Iran. *The First Conference on Integrative Zoology*. Iran: Ferdowsi University of Mashhad (2012).
14. Wanrong Z. The succession of rodent populations in Erdosi desert-grasslands and the multiplicity of the reservoirs in plague natural foci. *Chin J Vector Biol Control* (1991) **2**:257–60.
15. Cully JF, Williams ES. Interspecific comparisons of sylvatic plague in prairie dogs. *J Mammal* (2001) **82**:894–905. doi:10.1644/1545-1542(2001)082<0894:ICOSPI>2.0.CO;2
16. Bearden SW, Brubaker RR. Recent findings regarding maintenance of enzootic variants of *Yersinia pestis* in sylvatic reservoirs and their significance in the evolution of epidemic plague. *Vector Borne Zoonotic Dis* (2010) **10**(1):85–92. doi:10.1089/vbz.2009.0043
17. Lorange EA, Race BL, Sebbane F, Hinnebusch BJ. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. *J Infect Dis* (2005) **191**(11):1907–12. doi:10.1086/429931
18. Brubaker RR. Factors promoting acute and chronic diseases caused by yersiniae. *Clin Microbiol Rev* (1991) **4**(3):309–24.
19. Duplantier JM, Duchemin JB, Chanteau S, Carniel E. From the recent lessons of the Malagasy foci towards a global understanding of the factors involved in plague reemergence. *Vet Res* (2005) **36**(3):437–53. doi:10.1051/vetres:2005007
20. World Health Organization. *Interregional Meeting on Prevention and Control of Plague*. Antananarivo: World Health Organization (2010).
21. Ben-Ari T, Neerinckx S, Gage KL, Kreppel K, Laudisoit A, Leirs H, et al. Plague and climate: scales matter. *PLoS Pathog* (2011) **7**(9):e1002160. doi:10.1371/journal.ppat.1002160
22. Eisen RJ, Gage KL. Adaptive strategies of *Yersinia pestis* to persist during inter-epizootic and epizootic periods. *Vet Res* (2009) **40**(2):1. doi:10.1051/vetres:2008039
23. Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* (1999) **96**(24):14043–8. doi:10.1073/pnas.96.24.14043
24. Cornelis GR, Boland A, Boyd AP, Geuijen C, Iriarte M, Neyt C, et al. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev* (1998) **62**(4):1315–52.
25. Revazishvili T, Rajanna C, Bakanidze L, Tsertsvadze N, Imnadze P, O'Connell K, et al. Characterisation of *Yersinia pestis* isolates from natural foci of plague in the Republic of Georgia, and their relationship to *Y. pestis* isolates from other countries. *Clin Microbiol Infect* (2008) **14**(5):429–36. doi:10.1111/j.1469-0691.2008.01953.x
26. Beridze L, Chubinidze S, Tsertsvadze N, Tsintsadze L, Gaphrindashyili M, Katsitadze G. Characteristics of natural foci of plague existing on territory of Georgia. *Sakartvelos Sameditsino Moambe* (2004) **524**:22–8.
27. Chubinidze S, Katsitadze G, Tsertsvadze N, Eloshvili M, Tsanava S. [Dynamics of epizootic activity of natural foci of plague in Georgia]. *Georgian Med News* (2008) 163:47–53.
28. O'Connel KP, Skowronski EW, Sulakvelidez A, Bakanidze L. *Emerging and Endemic Pathogens: Advances in Surveillance, Detection and Identification*. 2010 ed. Netherlands: Springer (2010). 175 p.
29. Cummings CA, Bormann Chung CA, Fang R, Barker M, Brzoska P, Williamson PC, et al. Accurate, rapid and high-throughput detection of strain-specific polymorphisms in *Bacillus anthracis* and *Yersinia pestis* by next-generation sequencing. *Investig Genet* (2010) **1**(1):5. doi:10.1186/2041-2223-1-5
30. Rajanna C, Revazishvili T, Rashid MH, Chubinidze S, Bakanidze L, Tsanava S, et al. Characterization of pPCP1 plasmids in *Yersinia pestis* strains isolated from the former soviet union. *Int J Microbiol* (2010) **2010**:760819. doi:10.1155/2010/760819
31. Tong ZZ, Zhou DS, Song YJ, Zhang L, Pei D, Han YP, et al. Genetic variations in the pgm locus among natural isolates of *Yersinia pestis*. *J Gen Appl Microbiol* (2005) **51**(1):11–9. doi:10.2323/jgam.51.11
32. You YH, Wang P, Wang YH, Zhang MJ, Song ZZ, Hai R, et al. Comparative genomic analysis of gene variations of two Chinese *Yersinia pestis* isolates from vaccine strain EV76. *Biomed Environ Sci* (2012) **25**(4):440–8. doi:10.3967/0895-3988.2012.04.009
33. Rudolph AE, Stuckey JA, Zhao Y, Matthews HR, Patton WA, Moss J, et al. Expression, characterization, and mutagenesis of the *Yersinia pestis* murine toxin, a phospholipase D superfamily member. *J Biol Chem* (1999) **274**(17):11824–31. doi:10.1074/jbc.274.17.11824
34. Cornelis GR. Yersinia type III secretion: send in the effectors. *J Cell Biol* (2002) **158**(3):401–8. doi:10.1083/jcb.200205077
35. Perry RD, Harmon PA, Bowmer WS, Straley SC. A low-Ca2+ response operon encodes the V antigen of *Yersinia pestis*. *Infect Immun* (1986) **54**(2):428–34.
36. Price SB, Straley SC. lcrH, a gene necessary for virulence of *Yersinia pestis* and for the normal response of *Y. pestis* to ATP and calcium. *Infect Immun* (1989) **57**(5):1491–8.
37. Jeannin P, Bottazzi B, Sironi M, Doni A, Rusnati M, Presta M, et al. Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. *Immunity* (2005) **22**(5):551–60. doi:10.1016/j.immuni.2005.03.008
38. Solomonia R, Nozadze M, Mikeladze E, Zhgenti E, Chanturia G, Kekeklidze M, et al. Characterization of atypical *Y. pestis* strains isolated in natural foci of Georgia. In *Abstracts of 112th ASM General Meeting*. San-Francisco (2012). p. 99.
39. Knowlton KU, Jeon ES, Berkley N, Wessely R, Huber S. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. *J Virol* (1996) **70**(11):7811–8.
40. Zauberman A, Cohen S, Mamroud E, Flashner Y, Tidhar A, Ber R, et al. Interaction of *Yersinia pestis* with macrophages: limitations in YopJ-dependent apoptosis. *Infect Immun* (2006) **74**(6):3239–50. doi:10.1128/iai.00097-06
41. Chromy BA, Choi MW, Murphy GA, Gonzales AD, Corzett CH, Chang BC, et al. Proteomic characterization of *Yersinia pestis* virulence. *J Bacteriol* (2005) **187**(23):8172–80. doi:10.1128/JB.187.23.8172-8180.2005
42. Rajanna C, Ouellette G, Rashid M, Zemla A, Karavis M, Zhou C, et al. A strain of *Yersinia pestis* with a mutator phenotype from the Republic of Georgia. *FEMS Microbiol Lett* (2013) **343**(2):113–20. doi:10.1111/1574-6968.12137
43. Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, et al. Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc Natl Acad Sci U S A* (2004) **101**(51):17837–42. doi:10.1073/pnas.0408026101
44. Knight SD. Structure and assembly of *Yersinia pestis* F1 antigen. *Adv Exp Med Biol* (2007) **603**:74–87. doi:10.1007/978-0-387-72124-8_6

45. Williamson ED, Oyston PC. Protecting against plague: towards a next-generation vaccine. *Clin Exp Immunol* (2013) **172**(1):1–8. doi:10.1111/cei.12044
46. Sebbane F, Jarrett C, Gardner D, Long D, Hinnebusch BJ. The *Yersinia pestis* caf1M1A1 fimbrial capsule operon promotes transmission by flea bite in a mouse model of bubonic plague. *Infect Immun* (2009) **77**(3):1222–9. doi:10.1128/IAI.00950-08
47. Taylor DE. Bacterial tellurite resistance. *Trends Microbiol* (1999) **7**(3):111–5. doi:10.1016/S0966-842X(99)01454-7
48. Calderon IL, Arenas FA, Perez JM, Fuentes DE, Araya MA, Saavedra CP, et al. Catalases are NAD(P)H-dependent tellurite reductases. *PLoS One* (2006) **1**:e70. doi:10.1371/journal.pone.0000070
49. Ponnusamy D, Hartson SD, Clinkenbeard KD. Intracellular *Yersinia pestis* expresses general stress response and tellurite resistance proteins in mouse macrophages. *Vet Microbiol* (2011) **150**(1–2):146–51. doi:10.1016/j.vetmic.2010.12.025
50. Wang W, Li GW, Chen C, Xie XS, Zhuang X. Chromosome organization by a nucleoid-associated protein in live bacteria. *Science* (2011) **333**(6048):1445–9. doi:10.1126/science.1204697
51. Philip NH, Brodsky IE. Cell death programs in *Yersinia* immunity and pathogenesis. *Front Cell Infect Microbiol* (2012) **2**:149. doi:10.3389/fcimb.2012.00149
52. Ye Z, Gorman AA, Uittenbogaard AM, Myers-Morales T, Kaplan AM, Cohen DA, et al. Caspase-3 mediates the pathogenic effect of *Yersinia pestis* YopM in liver of C57BL/6 mice and contributes to YopM's function in spleen. *PLoS One* (2014) **9**(11):e110956. doi:10.1371/journal.pone.0110956
53. Bearden SW, Fetherston JD, Perry RD. Genetic organization of the yersiniabactin biosynthetic region and construction of avirulent mutants in *Yersinia pestis*. *Infect Immun* (1997) **65**(5):1659–68.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 June 2016
doi: 10.3389/fmicb.2016.01022

Epizootology and Molecular Diagnosis of Lumpy Skin Disease among Livestock in Azerbaijan

Shalala Zeynalova[1], Kliment Asadov[2], Fizuli Guliyev[1], Mahira Vatani[1] and Vidadi Aliyev[3]*

[1] Virology Department, Republican Veterinary Laboratory, Baku, Azerbaijan, [2] Sector for Epidemiological Control, Treatment, and Prevention of Especially Dangerous Pathogens, State Veterinary Control Service, Baku, Azerbaijan, [3] Jalilabad Rayonal Veterinary Office, Jalilabad, Azerbaijan

Lumpy skin disease (LSD) is a viral disease of livestock that can cause cutaneous and internal lesions, affecting milk production, hide quality and in some cases death of the infected animal. After an outbreak in neighboring Iran, a working group from the Azerbaijan State Veterinary Control Service was sent to the border rayons (administrative districts) to determine if any cattle in southern Azerbaijan were infected. The Rayonal Veterinary Offices were contacted to look for and report any cases of LSD in their rayons. Animals exhibiting clinical signs consistent with LSD infection were first observed in the rayon of Bilasuvar and more cases were subsequently identified in Jalilabad, Ujar, and Aghdash rayons. Samples were collected from blood, and/or lesions of suspected infected animals and internal organs of cattle that died and were tested at the Republican Veterinary Laboratory in Baku using real-time polymerase chain reaction (PCR). From June to November 2014, 2,762 cattle in Azerbaijan were reported to have clinical signs or gross necropsy lesions consistent with LSD. Of 269 samples tested for LSD virus by real-time PCR, 199 (74%) were positive. A total of 33 cattle died, which was 1.2% of those exhibiting clinical signs of disease. Samples from nodular cutaneous lesions were more frequently positive by PCR and had higher concentrations of virus than blood and pooled internal organ samples. Preventative measures including movement restrictions, vector control and vaccination were put into place to slow the spread of disease. Ongoing surveillance should continue as environmental persistence of the virus may lead to further outbreaks of disease.

Keywords: lumpy skin disease, pox virus, cattle, Azerbaijan, PCR

OPEN ACCESS

Edited by:
Jeanne Marie Fair, Defense Threat Reduction Agency, USA

Reviewed by:
Nick Lyons, The Pirbright Institute, UK
Ankit Shukla, Technical University of Denmark, Denmark

****Correspondence:***
Shalala Zeynalova
zeynalova.shelale@rambler.ru

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *19 June 2015*
Accepted: *15 June 2016*
Published: *29 June 2016*

Citation:
Zeynalova S, Asadov K, Guliyev F, Vatani M and Aliyev V (2016) Epizootology and Molecular Diagnosis of Lumpy Skin Disease among Livestock in Azerbaijan. Front. Microbiol. 7:1022. doi: 10.3389/fmicb.2016.01022

INTRODUCTION

Lumpy skin disease (LSD) is a disease of livestock caused by lumpy skin disease virus (LSDV), a DNA virus belonging to the genus *Capripox* in the *Poxviridae* family. Although other strains of *Capripox* infect sheep and goats, LSDV is associated with cattle (Davies, 1981). Lumpy skin disease was first recorded in Zambia in 1929, and then spread throughout southern Africa and north to Sudan. It was first diagnosed outside of Africa in Israel in 1989 and in subsequent years, cases were reported in Bahrain, Kuwait, Oman, Yemen, Lebanon, and Jordan (Wainwright et al., 2013). The disease is characterized by fever and nodular lesions on the skin, mucous membranes, and internal organs (Prozesky and Barnard, 1982; OIE Terrestrial Manual, 2010). Reduction in milk production, damaged hides, temporary or permanent

FIGURE 1 | Rayons with suspect or confirmed LSDV infections in cattle reported during 2014.

sterility, and/or death of infected animals may occur, resulting in significant economic consequences in affected countries (Sajid et al., 2012; Alemayehu et al., 2013). Severity of clinical signs depends on the strain of the virus and breed of infected cattle (OIE Terrestrial Manual, 2010). Transmission is primarily by indirect contact via biting insects (Kitching and Mellor, 1986; Chihota et al., 2001; Magori-Cohen et al., 2012). The average incubation period of LSDV is six to nine days (OIE Terrestrial Manual, 2010). The average mortality rate of the disease is approximately 10%, although the mortality rate is often higher in secondary infections (OIE Terrestrial Manual, 2010).

Azerbaijan is situated on the Caspian Sea and shares a border with Russia to the north, Georgia to the northwest, Armenia to the west, and Iran to the south. Azerbaijan is divided into rayons (or administrative districts), and cities. Each rayon and city has a Rayonal Veterinary Office (RVO) that provides veterinary and epidemiologic support for the control of animal diseases in the rayon. Each rayon also has field veterinarians stationed throughout the rayon who report to the RVO. Each RVO reports any cases of reportable diseases to the State Veterinary Control Service (SVCS).

Outbreaks of LSD were reported to the OIE in Turkey and Iraq in late 2013 and in Iran in early 2014 (OIE World Animal Health Information Database [WAHID]; Wainwright et al., 2013; European Food Safety Authority [EFSA], 2015). The objective of the investigation was to determine if LSDV had spread into Azerbaijan from neighboring countries reporting cases. In May 2014, an outbreak response team was sent from the SVCS in Baku to Azerbaijan's southern border area to examine cattle and to make control and prevention recommendations. This paper describes the first confirmed cases of LSD in Azerbaijan in 2014 (OIE, 2014) as well as the diagnostic methods used and control and prevention measures applied.

MATERIALS AND METHODS

Affected Areas and Populations

In May 2014, a working group composed of clinicians and veterinary epidemiologists from the SVCS and local field veterinarians investigated small cattle farms in Bilasuvar rayon after reports of LSD were reported in the neighboring country of Iran. Bilasuvar is a common area of animal movement near

the Iranian border. The veterinary epidemiologist from the RVO in Bilasuvar asked 35 field veterinarians stationed throughout the rayon to visit all farms in their areas to inquire about any suspected cases of LSD. A registration system of farms was not in place at the time, so identification of farms was based on the local knowledge of the field veterinarians. Cattle exhibiting clinical signs consistent with LSD were identified in the village of Amankend. Upon confirmation of suspect cases, every RVO in Azerbaijan was notified of the outbreak. In turn, the RVOs instructed field veterinarians across the country to report any suspect cases and to collect samples to be sent to the Republican Veterinary Laboratory for confirmatory testing. If positive cases were observed, veterinarians were instructed to visit those farms daily until the cases resolved or the animals died. Total case numbers were shared with the SVCS on a monthly basis. Cases of presumptive LSD were subsequently reported in the region of Jalilabad, which also borders Iran, and in Ujar and Aghdash, which are more centrally located within the country but lie along major roadways that are connected to southern Azerbaijan (**Figure 1**).

Animal and Tissue Sampling

Field veterinarians were responsible for collecting biological specimens from affected animals according to established SVCS protocols and guidance provided by the SVCS at the time of the outbreak. Samples were sent to the Virology Department at the Republican Veterinary Laboratory for testing. A skin scrape of nodular skin lesions and 5 mL blood samples in EDTA were collected from the necks and heads of affected animals. Necropsies were performed by field or RVO veterinarians on 33 dead cattle exhibiting lesions consistent with LSD; small portions of lung, kidney, liver, and heart were collected from these animals for testing.

Samples were prepared according to SVCS protocol #475 (State Veterinary Control Service of Azerbaijan, 2008). Skin samples were placed into phosphate buffered saline (1X PBS) for one hour before DNA extraction. A piece (approximately 1.3 cm^2) of each of the internal organs (liver, kidney, heart, and lung) was cut into small pieces using scissors and pooled together (up to 5 g total). Samples (skin or pooled internal organs) were homogenized using a mortar and pestle with a small amount of ground glass and 8 mL 1× PBS. Once homogenized, the slurry was decanted into 15 mL conical tubes and centrifuged at 8,000 rpm for ten minutes. DNA was extracted from 140 μL aliquots of the resulting supernatant using DNeasy Blood & Tissue Mini Kits (Qiagen, USA) according to the manufacturer's instructions. These kits were also used to extract DNA from blood samples. Mortar and pestles were disinfected with bleach between samples. The study described was an example of outbreak surveillance conducted with approval of the Azerbaijan State Veterinary Control Service (SVCS) Scientific Committee, which reviewed the activities for scientific and ethical concerns for the use of animals.

Appropriate biosafety protocols were followed during sampling and laboratory work to ensure personnel, environmental and animal safety. Personnel wore personal protective equipment (PPE) and all instruments were disinfected between samples. Waste materials were properly treated and discarded.

TABLE 1 | Primer, probe, and positive template control sequences for the LSDV real-time PCR assay.

Primer/probe/PTC	Sequence
Forward primer	5′-TCC-GAG-CTC-TTT-CCT-TAC-TAT-3′
Reverse primer	5′-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3′
Probe[a]	5′6FAM-CAATGGGTAAAAGATTTCTA - MGBNFQ 3′
Positive Template Control	5′ ATG GCG ATG TCC ATT CCC TGA CCA ATG GGT AAA AGA TTT CTA TCG TAA CAG ATG AAA GAG CAA GCT ACT ATT CCT CAC GGA AAT GAA ATG CTT C 3′

[a]*Fluorescent dye abbreviation: 6FAM = 6-Carboxyfluorescein; MGBNFQ = Minor groove binding, non-fluorescent quencher.*

Real-Time PCR Testing

A real-time PCR assay was used to rapidly diagnose cases. Specific forward and reverse capripox primers were used as described by Balinsky et al. (2008); sequences are listed in **Table 1**. Primers, probe and positive template control materials were provided by Dr. Ketan Patel of the Naval Medical Research Center in Ft. Detrick, Maryland.

FIGURE 2 | Nodules on head area.

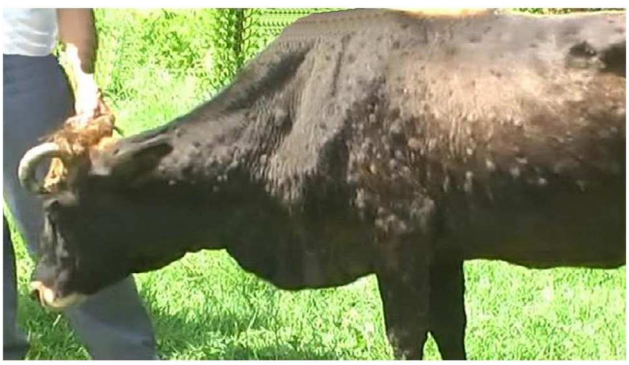

FIGURE 3 | Nodules on neck and abdominal area.

TABLE 2 | Clinical diagnosis of LSD in cattle by village and farm.

Rayon	Date of outbreak onset	Date of last case in village	Village	Estimated # of cattle	# Suspected cases	Apparent morbidity % (95% CI)	# Deaths	Apparent case fatality (%)	# Farms in village	# of Farms with LSD	% of Farms affected (95% CI)
Bilasuvar	June 07, 2014	June 21, 2014	Amankend	926	128	13.8 (11.7–16.2)	0	0	39	4	10.0 (3.3–25.1)
Jalilabad	July 08, 2014	July 24, 2014	Alar	3,142	114	3.6 (3.0–4.3)	0	0	32	4	12.5 (4.08–30)
Aghdash	October 04, 2014	October 25, 2014	Pirkeke	3,863	126	3.3 (2.7–3.8)	2	1.6	43	8	18.6 (9–34)
	October 04, 2014	November 01, 2014	Kukel	967	123	12.7 (10.7–15.0)	2	1.6	44	7	15.9 (7.15–30.6)
	October 04, 2014	October 22, 2014	Arabkendi	1,299	127	9.8 (8.2–11.5)	2	1.6	35	6	17.1 (7.16–34.3)
	October 15, 2014	November 05, 2014	Shakili	901	124	13.7 (11.6–16.2)	1	0.8	31	5	16.1 (6.1–34.4)
	October 05, 2014	November 29, 2014	Shansabd	1,342	137	10.2 (8.7–11.9)	3	2.2	44	7	15.9 (7.15–30.6)
Ujar	October 02, 2014	October 25, 2014	Elekend	3,519	287	8.1 (7.3–9.1)	3	1.0	31	5	16.1 (6.1–34.47)
	October 02, 2014	October 28, 2014	Leke	4,884	244	5.0 (4.4–5.6)	4	1.6	48	6	12.5 (5.1–26)
	October 03, 2014	November 01, 2014	Yuxarishilyan	4,921	276	5.6 (5.0–6.2)	3	1.1	51	8	15.7 (7.5–29.1)
	October 03, 2014	October 30, 2014	Alpoud	4,670	271	5.8 (5.2–6.5)	5	1.8	48	5	10.4 (4.53–22.1)
	October 03, 2014	October 25, 2014	Tezeshilyan	4,772	283	6.0 (5.3–6.6)	2	0.7	54	6	11.1 (4.6–23.3)
	October 04, 2014	November 02, 2014	Qaraqumlar	3,878	268	6.9 (6.1–7.7)	3	1.1	49	8	16.3 (7.8–30.2)
	October 05, 2014	October 27, 2014	Boyad	3,674	254	6.9 (6.1–7.8)	3	1.2	43	6	14.0 (5.8–28.6)
	TOTAL			**42,758**	**2,762**	**6.5 (6.2–6.7)**	**33**	**1.2**	**592**	**85**	**14.3 (11.7–17.5)**

PCR master mix was prepared using Taq polymerase, 10× PCR buffer, 50 mM $MgCl_2$, and 10 mM dNTPs according to the protocol described by Balinsky et al. (2008). A total of 5 μL of extracted sample DNA or template controls were added to 15 μL of the prepared master mix for a total volume of 20 μL. The reaction was run on a LightCycler 2.0 PCR instrument (Roche Diagnostics, Germany) using the thermocycling conditions described by Balinsky et al. (2008). Before sample testing, the positive template control (PTC) was serially diluted from 1 pg/μL to 0.001 fg/μL and run on the R.A.P.I.D. PCR instrument (BioFire Defense, Salt Lake City, UT, USA). The resultant cycle threshold (C_T) vs. log of the PTC concentration graph gave a slope of 3.76, corresponding to 92% amplification efficiency (with 100% doubling every cycle); this was sufficient for continued testing of samples. Based on the standard curve, PTC at a concentration of 1 fg/μL was used in subsequent measurements.

In order to determine the best sample to collect for testing of future cases, a comparison of PCR results was undertaken in the 33 animals that died where three sample types were tested including nodular lesions, blood and pooled internal organ samples as well as in the 27 animals that had paired blood and nodular lesion samples.

Control Measures

Treatment of sick animals varied by case, but typically included disinfection of cutaneous lesions using iodine and treatment of secondary bacterial infections with sulfanilamide. All field veterinarians wore PPE while on animal premises and while handling animals including disposable gowns, rubber boots, gloves and head covers. All reusable instruments were disinfected between premises and waste materials were properly treated and discarded. Preventative measures were enacted, including movement control of animals as well as restriction of vehicle access to affected farms. Farms with affected animals were visited daily by the field veterinarians until the cases either died or recovered. Neighboring countries were notified of the presence of LSD in Azerbaijan and vaccines were ordered for a targeted vaccination campaign.

RESULTS

Affected Area and Populations

Cattle exhibiting clinical signs consistent with LSD were reported in Bilasuvar, Jalilabad, Ujar, and Aghdash rayons (**Figure 1**). Affected cattle refused food and exhibited fevers, purulent oculonasal discharge, and malaise. In some cases, red, firm nodules were observed along the neck and abdomen of the animal, with degenerative changes noted on the skin surface around the nodules, such as necrotic areas, edema, and exudate (**Figures 2** and **3**). A pulmonary form resulting in shortness of breath was also documented, with the majority of cases reported in October 2014. Death in the pulmonary cases was presumed to have resulted from asphyxia. Lung congestion and nodules throughout internal organs were often observed during necropsies.

A total of 2,762 cases were reported and 33 cattle (1.2%) died (**Table 2**). Overall, about 6.5% of cattle in the affected villages were reported as positive to the SVCS with a 95% confidence interval of 6.2–6.7% (**Table 2**). A total of 14% of farms in those villages reported at least one case of LSD. Cases were reported in June, July, October, and November 2014 (**Table 2**).

Real-Time PCR Testing

A total of 269 samples were tested by real-time PCR for the presence of LSDV from 176 animals, including 130 skin samples, 106 blood samples, and 33 internal organ pools (**Table 3**). A total of 199 (74%) samples were positive by PCR. All skin lesions tested were positive and had lower C_T values than blood or organ samples, suggesting higher concentrations of virus. Blood had the highest average C_T value and was least likely to be positive, suggesting lower concentrations of virus.

In the 33 animals that died, nodular lesions tested positive in all 33 cases (100%); 27 of 33 (82%) pooled internal organ samples tested positive while only 13 of 33 (39%) blood samples tested positive. Paired blood and nodular lesion samples were submitted for 27 suspect cases. Of these, all 27 nodular lesion samples (100%) were positive while only 11 blood samples (41%) tested positive.

Control Measures

Overall, the majority of suspected infected cattle recovered, although it is unclear which, if any, treatment regimens contributed to recovery. All affected farms were instructed to restrict animal movement off the farm for 30 days from the time the last case was identified. Ectoparasiticides were applied to healthy ruminants on the infected farms and on surrounding farms where outbreaks occurred. One of three locally available ectoparasiticides was used to spray animals, including Ektosan (Brovafarma Ltd, Ukraine), Blotic 7% Emulsion (Topkim, Turkey) or Butox (MSD Animal Health, India). Dilutions were made according to manufacturer's recommendations and farmers were asked to apply the ectoparasiticide twice weekly. After the outbreak, two million doses of live sheep and goat pox vaccine (Poxvac, Vetal Company, Turkey) were purchased. In 2015, a targeted 5-year vaccination campaign was initiated to control the spread of this disease in Azerbaijan. A total of 1.6 million cattle in the affected rayons, neighboring rayons, and rayons on the southern Azerbaijan border were vaccinated in 2015 with

TABLE 3 | Real-time PCR testing results.

Type of Sample	# Tested	# Positive	% Positive	Average C_T value	Standard deviation of C_T values
Skin Lesion	130	130	100	19.3	1.10
Blood	106	42	40	29.4	1.39
Internal Organ pool	33	27	82	22.9	0.65
TOTAL	**269**	**199**	**74**	**–**	**–**

Samples are from 176 animals (33 were tested for all three samples, 27 had skin lesion and blood samples submitted while the remaining only had skin or blood tested).

some vaccine held in reserve in the event of additional outbreaks. Cattle 3 months of age and over were included in the campaign with a focus on animals that migrate to summer pastures. For 2016–2019, approximately 15 million cattle are planned to be vaccinated throughout the country annually with 9 million cattle in high risk areas being vaccinated twice a year.

DISCUSSION

Although sheep and goat pox is considered endemic in Azerbaijan and the SVCS routinely conducts vaccinations against these diseases, LSDV had not been identified in Azerbaijan before the 2014 outbreak. Biting insects are thought to be responsible for transmitting this disease (Salib and Osman, 2011; Magori-Cohen et al., 2012) and may have introduced LSDV to the Bilasuvar region after crossing the border or being transported by vehicles into the rayon. Notably, the onset of this outbreak in early summer overlapped with periods of peak biting insect activity. It is also possible that the virus was introduced through the migration or movement of animals into Bilasuvar from an infected area, and was subsequently transmitted by direct or indirect contact. In support of this theory, Tuppurainen and Oura (2014) suggest that the infected animals could have originated in one of several nearby countries with LSD, such as Iran, Iraq, or Turkey. There is no conclusive evidence of either method of transmission. None of the farmers from where the first cases were observed reported animal movement from Iran or other LSD positive countries. The spread of infection within Azerbaijan may have resulted from movement of sale animals from Bilasuvar to the more northern rayons of Ujar and Aghdash. Farmers in the northern areas reported purchasing animals from the southern Azerbaijan rayons of Bilasuvar and Jalilabad, although specific farms were not identified. Additional characterization of the virus by sequencing may help to better determine the source of this outbreak. LSD outbreaks have since been reported to the OIE in Greece, Armenia, Georgia and Russia.

Overall, 6.5% of susceptible cattle in the affected areas were reported as having LSD and 1.2% of suspected infected cattle died during the 2014 outbreak in Azerbaijan. Official animal case definitions were not in place in Azerbaijan at the time of the outbreak, but have since been developed and formally adopted for future reporting purposes. It should be noted that not all suspected cases of LSD were confirmed to be infected by PCR, nor were other factors considered that may have caused animals to be more or less likely to die from infection. The apparent morbidity and case fatality rates reported from this outbreak are also subject to reporting biases as the estimates relied on passive surveillance. Variable mortality rates and case fatality rates for LSD have been observed in other countries. Mortality rates in an outbreak in Oman reached 13.6 and 15.4% in two locations (Tageldin et al., 2014), while a mortality rate of 2% was reported among cattle in six feedlot operations in Ethiopia (Alemayehu et al., 2013). An analysis of active and historic outbreaks of LSD in Ethiopia revealed mortality rates between 3.4 and 5.9% (Ayelet et al., 2014). In Jordan, a study assessing the efficacy of vaccination reported 10% mortality among unvaccinated cattle and a case fatality rate of 24% (Abutarbush, 2014). The low apparent case fatality rate of 1.2% found in this outbreak could be a true low case fatality rate, or could be a result of poor follow-up of cases by field veterinarians or under-reporting to the SVCS.

Real-time PCR is a rapid, sensitive and specific method for confirmation of capripoxviruses including LSD (Balinsky et al., 2008). In this investigation, two-thirds of all samples tested from suspect animals were positive for the presence of viral DNA. Skin nodule samples consistently tested positive for LSDV; blood and organ samples were less likely to test positive. This aligns with the results of a study that found that LSD viremia is relatively short-lived – blood samples were positive for PCR for 4–11 days post-infection, while virus could be detected in skin lesions up to 92 days post-infection (Tuppurainen et al., 2005). In addition, on average, skin nodule samples in this study exhibited a higher concentration of virus than other samples, as evidenced by the lower average C_T values observed in PCR testing. Quantitative real-time PCR assays were not performed, so the viral load of the different sample types could not be estimated.

Since the virus is very stable in the environment and can be transmitted by insects, mass vaccination of livestock is required to control the spread of disease (Wainwright et al., 2013; Tuppurainen and Oura, 2014). Other countries, such as Israel and Lebanon, have successfully controlled outbreaks with vaccination (Tuppurainen and Oura, 2014). Sheep and goat pox virus vaccines have been widely used against LSDV in cattle because the capripox viruses tend to be host-specific, yet offer cross-protection within the *Capripoxvirus* genus when vaccinations are administered (OIE Terrestrial Manual, 2010; Tuppurainen et al., 2014). The vaccines purchased for use in Azerbaijan's vaccination program were advertised as a sheep–goat virus, which should offer cattle immunity against LSDV. Ongoing vaccination of cattle in the affected and surrounding areas will be necessary to keep cattle protected against exposure to the virus through the environment and biting insects. Vector control is also an important aspect of limiting spread of disease and should be used during active outbreaks.

Lumpy skin disease virus was detected for the first time in Bilasuvar rayon in Azerbaijan in 2014 and subsequently identified in three other rayons. Control measures were implemented, including restricted animal movement, vector control and a vaccination campaign. No additional cases were reported after November 2014. However, environmental persistence of the virus will likely continue to pose a risk to unvaccinated cattle in the affected rayons. Ongoing passive surveillance will continue to look for new cases throughout the country.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENT

The authors are grateful to Ketan Patel and Matthew McGillicuddy of the Naval Medical Research Center at Fort

Detrick, Maryland, for generously providing the reagents for the assay, Natiq Javadov from Azerbaijan State Veterinary Control Service for assistance with ArcGIS and to the Cooperative Biological Engagement Program personnel in Azerbaijan: Sebastian McClendon for technical assistance with the Capripox assay, and Lisa Strelow, April Johnson, Saida Aliyeva, and Sonya Narodny for support with publication review.

REFERENCES

Abutarbush, S. (2014). Efficacy of vaccination against lumpy skin disease in jordanian cattle. *Vet. Rec.* 175:302. doi: 10.1136/vr.102271

Alemayehu, G., Zewde, G., and Admassu, B. (2013). Risk assessments of lumpy skin diseases in borena bull market chain and its implication for livelihoods and international trade. *Trop. Anim. Health Prod.* 45, 1153–1159. doi: 10.1007/s11250-012-0340-9

Ayelet, G., Haftu, R., Jemberie, S., Belay, A., Gelaye, E., Sibhat, B., et al. (2014). Lumpy skin disease in cattle in central Ethiopia: outbreak investigation and isolation and molecular detection of the virus. *Rev. Sci. Tech.* 33, 877–887. doi: 10.20506/rst.33.3.2325

Balinsky, C., Delhon, G., Smoliga, G., Prarat, M., French, R., Geary, S., et al. (2008). Rapid preclinical detection of sheeppox virus by a real-time PCR assay. *J. Clin. Microbiol.* 46, 438–442. doi: 10.1128/JCM.01953-07

Chihota, C., Rennie, L., Kitching, R., and Mellor, S. (2001). Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae). *Epidemiol. Infect.* 126, 317–321. doi: 10.1017/S0950268801005179

Davies, F. (1981). "Sheep and goat pox and lump skin diseases," in *Virus disease of Food Animals*, ed. E. P. J. Gibbs (London: Academic Press).

European Food Safety Authority [EFSA] (2015). Scientific opinion on lumpy skin disease; EFSA panel on animal health and welfare (AHAW). *EFSA J.* 13:3986. doi: 10.2903/j.efsa.2015.3986

Kitching, R., and Mellor, P. (1986). Insect transmission of capripoxvirus. *Res. Vet. Sci.* 40, 255–258.

Magori-Cohen, R., Louzoun, Y., Herziger, Y., Oron, E., Arazi, A., Tuppurainen, E., et al. (2012). Mathematical modelling and evaluation of the different routes of transmission of lumpy skin disease virus. *Vet. Res.* 43:1. doi: 10.1186/1297-9716-43-1

OIE (2014). *Report on Immediate Notification of LSDV in Azerbaijan 7 July 2014*. Available at: http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=15632

OIE Terrestrial Manual (2010). *Lumpy Skin Disease, Chapter 2.4.1 4.* Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.14_LSD.pdf (accessed May 14, 2015).

OIE World Animal Health Information Database [WAHID]. *OIE World Animal Health Information Database*. Available at: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasehome (accessed May 26, 2015).

Prozesky, L., and Barnard, B. J. (1982). A study of the pathology of lumpy skin disease in cattle. *Onderstepoort J. Vet. Res.* 49, 167–175.

Sajid, A., Chaudhary, Z., Sadique, U., Maqbol, A., Anjum, A., Qureshi, M., et al. (2012). Prevalence of goatpox disease in Punjab province of Pakistan. *J. Anim. Plant Sci.* 22, 28–32.

Salib, F., and Osman, A. (2011). Incidence of lumpy skin disease among Egyptian cattle in Giza Governorate, Egypt. *Vet. World* 4,162–167.

State Veterinary Control Service of Azerbaijan (2008). *Preparation of Samples from Tissue # V-04/M.M.-016/1.0. Protocol #475*. Azerbaijan: State Veterinary Control Service of Azerbaijan

Tageldin, M., Wallace, D., Gerdes, G., Putterill, J., Greyling, R., Phosiwa, M., et al. (2014). Lumpy skin disease of cattle: an emerging problem in the Sultanate of Oman. *Trop. Anim. Health Prod.* 46, 241–246. doi: 10.1007/s11250-013-0483-3

Tuppurainen, E., Eeva, S., Pearson, C., Bachanek-Bankowska, K., Knowles, N., Amareen, S., et al. (2014). Characterization of sheep pox virus vaccine for cattle against lumpy skin disease virus. *Antivir. Res.* 109, 1–6. doi: 10.1016/j.antiviral.2014.06.009

Tuppurainen, E., and Oura, C. (2014). Lumpy skin disease: an African cattle disease getting closer to the EU. *Vet. Rec. 175,* 300–301. doi: 10.1136/vr.g5808

Tuppurainen, E., Venter, E., and Coetzer, J. (2005). The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort J. Vet. Res.* 72, 153–164. doi: 10.4102/ojvr.v72i2.213

Wainwright, S., El Idrissi, A., Mattioli, R., Tibbo, M., Njeumi, F., and Raizman, E. (2013). Emergence of lumpy skin disease in the Eastern Mediterranean Basin countries. *FAO Empres Watch.* 29, 1–6.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 16 February 2016
doi: 10.3389/fpubh.2016.00008

Flaviviruses as a Cause of Undifferentiated Fever in Sindh Province, Pakistan: A Preliminary Report

*Erum Khan[1,2], Joveria Q. Farooqi[1,2], Kelli L. Barr[3], Dhani Prakoso[3], Amna Nasir[1,2], Akbar Kanji[1,2], Sadia Shakoor[1,2], Faisal Riaz Malik[1,2], Rumina Hasan[1,2], John A. Lednicky[4] and Maureen T. Long[3]**

[1] Department of Pathology, Aga Khan University, Karachi, Pakistan, [2] Department of Microbiology, Aga Khan University, Karachi, Pakistan, [3] Emerging Diseases and Arbopathogens Research and Testing Laboratory, Department of Infectious Diseases and Pathology, University of Florida, Gainesville, FL, USA, [4] Department of Environmental and Global Health, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA

OPEN ACCESS

Edited by:
Nathan Wolfe,
Metabiota, USA

Reviewed by:
Lisa F. P. Ng,
Agency for Science Technology and Research (A*STAR), Singapore
Ankit Shukla,
Technical University of Denmark, Denmark

***Correspondence:**
Maureen T. Long
longm@ufl.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: 24 August 2015
Accepted: 17 January 2016
Published: 16 February 2016

Citation:
Khan E, Farooqi JQ, Barr KL, Prakoso D, Nasir A, Kanji A, Shakoor S, Malik FR, Hasan R, Lednicky JA and Long MT (2016) Flaviviruses as a Cause of Undifferentiated Fever in Sindh Province, Pakistan: A Preliminary Report. Front. Public Health 4:8. doi: 10.3389/fpubh.2016.00008

Arboviral diseases are expanding worldwide, yet global surveillance is often limited due to diplomatic and cultural barriers between nations. With human encroachment into new habitats, mosquito-borne viruses are also invading new areas. The actual prevalence of expanding arboviruses is unknown in Pakistan due to inappropriate diagnosis and poor testing for arboviral diseases. The primary objective of this study was to document evidence of flavivirus infections as the cause of undifferentiated fever in Pakistan. Through a cooperative effort between the USA and Pakistan, patient exposure to dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV) was examined in Sindh Province for the first time in decades. Initial results from the 2015 arbovirus season consisting of a cross-sectional study of 467 patients in 5 sites, DENV NS1 antigen was identified in 63 of the screened subjects, WNV IgM antibodies in 16 patients, and JEV IgM antibodies in 32 patients. In addition, a number of practical findings were made including (1) *in silico* optimization of RT-PCR primers for flavivirus strains circulating in the Middle East, (2) shipping and storage of RT-PCR master mix and other reagents at ambient temperature, (3) Smart phone applications for the collection of data in areas with limited infrastructure, and (4) fast and reliable shipping for transport of reagents and specimens to and from the Middle East. Furthermore, this work is producing a group of highly trained local scientists and medical professionals disseminating modern scientific methods and more accurate diagnostic procedures to the community.

Keywords: arbovirus, flavivirus, dengue viruses, Japanese encephalitis virus, West Nile virus, transboundary diseases

INTRODUCTION

Political boundaries do not constrain human, animal, or plant diseases. As humans and animals migrate across these borders, their associated diseases relocate in tandem. Newly encroaching diseases are often overlooked because of limited surveillance, political motivation, resources, and infrastructure (1). Collaboration of health and scientific professionals from different countries

occurs in a politically neutral atmosphere that can have rapid positive and sustainable impacts on human and animal health and the control of emerging diseases (2, 3). The growth in scientific personnel and infrastructure is essential to decrease the movement of diseases that threaten public health (1, 4–7). Many outbreaks since 1990 like prion disease in the UK, West Nile virus (WNV) in the America's, and avian H5N2 in Canada and the US have been economically destabilizing and highlight the need for transboundary collaboration (8).

In the past decade, mosquito-borne viral diseases have emerged in many new locales, rapidly attaining endemic status. In Pakistan, arboviral diseases are frequently overlooked or misdiagnosed because of the vague symptoms and extensive differential diagnoses, which overlap with many other pathologies, such as Crimean-Congo Hemorrhagic fever (CCHF), malaria, hepatitis C virus infection, Alkhurma virus, Kyasanur forest virus disease, rickettsiosis, ehrlichiosis, leptospirosis, typhoid fever, meningococcemia, borreliosis, Q fever, and influenza. Furthermore, manifestations of arboviral disease mimic other febrile diseases and severe disease can present as a hemorrhagic illness [dengue virus (DENV), yellow fever virus, Zika virus, and Lassa fever virus], neurological disease (WNV, DENV), or arthritis (chikungunya virus, Zika virus, and DENV) (9, 10). Because vaccines or antivirals do not exist for most of these viruses, surveillance becomes an essential part of control *via* detection and communication. The cornerstone of active and passive surveillance is accurate diagnostic assessment (9, 10).

There has been limited published data for arbovirus surveillance in Pakistan. Historically, only the presence of DENV subtypes 1 and 2 were detected in isolated outbreaks in Pakistan in the twentieth century (11, 12). Since 2005, all four subtypes of DENV have spread throughout the country (12–15). In neighboring Punjab province, the seroprevalence of DENV in patients was 42.63% in 2013 (16). The WHO lists Japanese encephalitis virus (JEV) as active in Pakistan (17), although most reports still indicate JEV activity mostly along the northern Pakistan–India border (10, 18–20). JEV is likely circulating in Pakistan at this time, but limited information exists regarding the actual disease burden JEV contributes to human health in Pakistan. In the early 2000s, 25% of the Pakistani military personnel who tested seropositive for JEV demonstrated cross-reactivity with WNV and thus a true determination of infection could not be verified (21). WNV has been detected in Pakistan since 1980s. Epidemiological work performed 20 years ago indicated that WNV antibodies were present in over 40% of the human population in Punjab province (21). Recently, a 55% seropositivity rate was detected in horses in Punjab province (18, 20–24). This high seroprevalence in horses suggests that WNV is also circulating in humans.

Described here are the initial data of a biological engagement program (BEP) implemented between Pakistan (Aga Khan University, Karachi, Pakistan) and the US (University of Florida, Gainesville, FL, USA) to perform a multisite study examining possible arboviral causes of febrile disease in citizens of Sindh province. *Via* a "train the trainer" format, this project aimed to provide Pakistani collaborators with training for virus surveillance and diagnostics in order to assess the prevalence of flaviviruses (DENV, WNV, and JEV) in Pakistan. The primary objective of this study was to document evidence of the above mentioned viral infections as causes of undifferentiated fever in order to build capacity for laboratory diagnosis and surveillance within Pakistan.

MATERIALS AND METHODS

A cross-sectional, observational study was performed to identify which arboviruses (DENV, WNV, and JEV) were the cause of acute undifferentiated febrile illness in selected basic health units and/or district hospitals of the Sindh region of Pakistan. A total of 1,000 patients (250/year) patients were targeted for enrollment under informed consent procedures that were reviewed and approved by the Ethics Review Committee, Aga Khan University (#3183-PAT-ERC-14) and the Institutional Review Board, University of Florida (#201500908). All enrolled subjects gave written informed consent in accordance with the Declaration of Helsinki. Patients were recruited with a case definition developed by the WHO and modified by the Pakistan Ministry of Health to incorporate syndromic findings of acute hemorrhagic fever, acute flaccid paralysis, and unexplained fever (25). Patient enrollment was performed during the monsoon season (May–October) during 2015. All patients, males and females between 10 and 50 years age meeting the case definition on the day of enrollment, were eligible for the study. Patients younger than 10 and older than 50 years of age and patients who tested positive for CCHV, influenza, malaria, tuberculosis, and bacterial septicemia during routine hospital admittance procedures were excluded (**Figure 1**). Briefly, all patients were tested for DENV antigen unless affected primarily by neurological abnormalities. If positive, serum was tested for DENV subtype by RT-PCR. All negative sera were tested *via* IgM capture ELISA for JEV and WNV.

Study Sites

Five study sites were established and personnel trained throughout the Sindh province in Pakistan (**Figure 2**). These sites included four medical colleges including Ghulam Mohammad Mahar Medical College (Sukkur, Pakistan), CMC Teaching Hospital (Larkana, Pakistan), and Muhammed Medical College Hospital (Mirpurkhas, Pakistan). Enrollment of study subjects was also established at a civil hospital in Hyderabad, Pakistan.

Data Collection and Processing Procedures

Originally, for communication within Pakistan between sites, networked computers were planned as the primary mode of reporting of test results. Connectivity was found to be a major issue; even if access was available, there were frequent interruptions and limited technological support. Android mobile phones provided an alternative for surveillance data collection and transmission (Epicollect®, http://www.epicollect.net/, Wellcome Trust, Imperial College London). At the study sites, patient information was collected on hard copy forms and de-identified

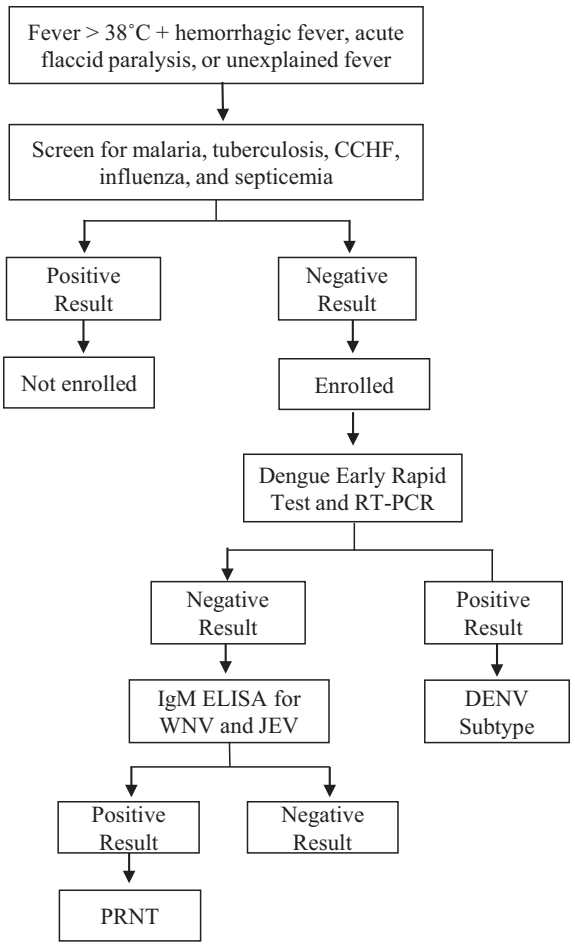

FIGURE 1 | Work flow for flavivirus exposure screening of enrolled patient samples.

data were stored on smart phones by a dedicated to a study medical officer and synced daily to a secure homepage on the *Epicollect* website.

Antigen and Antibody Screening Tests

Standard operating procedures were developed that follow published WHO/CDC guidelines or publications that were optimized for detecting Asian variants of arbovirus (25). Primary DENV screening in patients was performed using a commercially available antigen capture test (Panbio Dengue Early Rapid Test, Alere, Waltham, MA, USA). IgM capture ELISA testing was performed using commercial assays for WNV and JEV (InBios, Seattle, WA, USA) following the manufacturer's instructions.

Real-Time PCR

For detection of nucleic acids of DENV, primer sequences were constructed for stains circulating in Pakistan *via* addition of degenerate nucleotides (**Table 1**) (26). Primers, standards, and controls were developed using synthetic DNA targets of the various portions of the viral genomes (**Table 2**) and real-time PCR was performed using a commercial master mix (BioRad iTaq

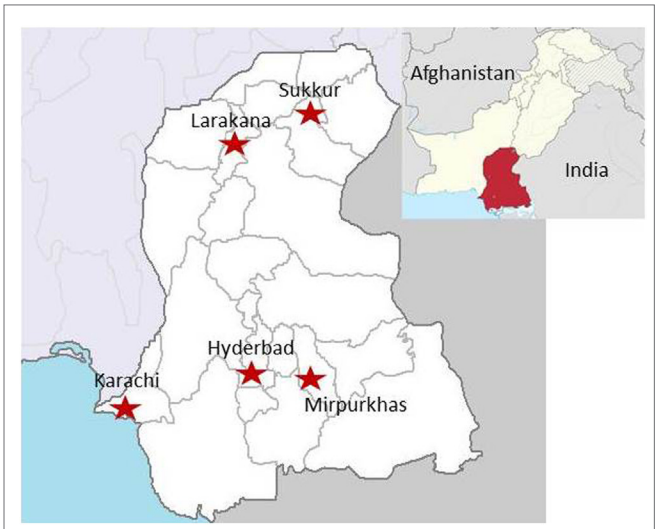

FIGURE 2 | Locations of study sites throughout Sindh, Pakistan. (Image obtained at https://en.wikipedia.org/wiki/Sindh#/media/File:Sindh_in_Pakistan_%28claims_hatched%29.svg)

Universal Probes Supermix, BioRad, Hercules, CA, USA) and a commercial real-time PCR machine (BioRad CFX 96, BioRad). All enrolled patients were screened for DENV *via* RT-PCR to identify DENV subtype.

RESULTS

Antigen and Antibody Screening Tests

As of this time, the five sites have enrolled a total of 467 participants. DENV antigen, using the DENV NS1 Early Rapid Test, was found in 63 of the enrolled patients (**Table 3**). WNV IgM antibodies were detected in 16 of 241 screened individuals and JEV IgM antibodies in 32 of 414 screened patients (**Table 3**). DENV was detected at all sights except Sukkur. Karachi had the highest DENV exposure with DENV antigen detected in 53 of 176 screened patients. Febrile patients with WNV exposure were found at four sites with eight of the patients living in Karachi, two in Hyderabad, two in Larkana, and one in Mirpurkhas (**Table 4**). JEV exposure was identified in 21 patients living in Karachi, 6 in Hyderabad, 1 in Mirpurkhas, 1 in Sukkur, and 1 patient in Larkana (**Table 4**).

A number of inconclusive samples were obtained from study subjects (**Table 3**). A high signal:noise ratio and cross-reactivity were factors that prevented adequate interpretation. In 16 of the 414 patients screened for JEV, the ELISA results fell above background noise and just below IgM positive. The WNV assays resulted in 14 of 241 samples with similar inconclusive readings. Cross-reactivity between the WNV and JEV ELISA assays was also an issue with 32 (up to 13%) samples that tested positive for both WNV and JEV exposure.

Real-Time PCR

Synthetic targets were developed for use as standards and controls for the RT-PCR platform (**Table 2**). Targets were optimized to perform as well as or better than conventional plasmids

TABLE 1 | RT-PCR primers and probes used for the detection of Dengue virus.

	Forward 5′–3′	Reverse 5′–3′	Probe 5′–3′
DENV 1	CAAAAGGAAGTCGYGCWATA	CTGAGTGAATTCTCTCTRCTRAAC	FAM–CATGTGGYTGGGAGCRCGC–BHQ-1
DENV 2	CAGGYTATGGCACYRTCACRAT	CCATYTGCAGCARCACSATCTC	FAM–CTCYCCRAGAACGGGCCTMGACTTCAA–BHQ-1
DENV 3	GGACTRGACACACGCACCCA	CATGTCTCTACCTTCTCGACTTGYCT	FAM–ACCTGGATGTCGGCTGAAGGAGCTTG–BHQ-1
DENV 4	TYRTYCTAATGATGCTRGTCG	TCCACCYGAGACTCCTTCCA	FAM–CGGAATGCGATGCGTAGGRGTAGGRA–BHQ-1

TABLE 2 | Synthetic DNA targets used for RT-PCR.

	Target gene	Target sequence
DENV 1	NS5	TTCGGAAAGGCAAAAGGAAGTCGTGCTATATGGTACATGTGGCTGGGAGCACGCTTTCTAGAGTTCGAAGCTCTTGGTTTCATGAACGAA GATCACTGGTTCAGCAGAGAGAATTCACTCAGCGGAGTGGAA
DENV 2	E	GCAGAGTTGACAGGCTATGGCACTGTCACGATGGAGTGCTCTCCGAGAACGGGCCTCGACTTCAATGAGATGGTGTTGCTGCAAATGGA AAATAAAGC
DENV 3	prM	TGTCGGCATGGGACTGGACACACGCACCCAAACCTGGATGTCGGCTGAAGGAGCTTGGAGGCAAGTCGAGAAGGTAGAGACATGGGC CCTTAGG
DENV 4	prM	ACTGTTTTCTTTGTCCTAATGATGCTAGTCGCCCCATCCTACGGAATGCGATGCGTAGGGGTAGGGAACAGAGACTTTGTGGAAGGAGTCT CGGGTGGAGCATGGGTCG

TABLE 3 | Patients testing positive for exposure to dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), or flavivirus (JEV–WNV cross-reactive) detected in patients enrolled in five study sites in Sind Province, Pakistan.

	Positive	Negative	Inconclusive/ borderline	Total samples	% Positive
JEV	32	367	16	414	7.73
WNV	16	211	14	241	6.64
DENV	63	404	0	467	13.49
Flavivirus	32	382	0	414	7.73

TABLE 4 | Patients testing positive for exposure to dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV) at each study site.

Study site	DENV	WNV	JEV
Karachi	53	8	21
Hyderabad	3	2	6
Mirpurkhas	6	4	1
Sukkur	0	0	1
Larkana	1	2	3

(**Figure 3**) and the difference in percent efficiency was 10% or less for DENV1 and 3 and <20% for DENV2 and DEN4. In addition, we found that our plasmid controls were frequently >100% in efficiency (slope <−3.5).

DISCUSSION

Arboviral infections have a global distribution; however, the burden of viral agents varies in different geographical regions. The true burden and epidemiology of arboviruses in Pakistan are not known as many of these infections, which present initially as a vague febrile illness, are often misdiagnosed.

The expansion of DENV in Pakistan has been notable in its intensity. Recently, DENV emerged in Karachi in Sindh, Pakistan affecting 3,640 patients with an estimated 40 deaths (12–14). WNV is an arbovirus undergoing expansion throughout the world. While it is similar to JEV in terms of syndromes, most human JEV exposure and illness is described in children (27). For WNV, people over the age of 55 years have the highest risk factors for neurological syndromes during the virus' recent expansion throughout Europe (28). Comparative analysis of risk for concomitantly circulating JEV and WNV has not been performed. Recent evidence demonstrates a high amount of WNV activity in Pakistan in horses (20); however, there is limited information regarding recent human exposure and, to the best of our knowledge, the most commonly reported cause of neurological arboviral disease would likely be JEV (20).

Dengue virus was the most frequently identified and widespread flavivirus detected in enrolled patients. These data show that DENV was detected in nearly one-third of all patients in Karachi while it was found at much lower rates in other locations. This is most likely due to the fact that Karachi is an expansive urban environment with the ideal climate for the DENV vector *Aedes aegypti*. The other four sites displayed a much lower human exposure of arboviral diseases than Karachi, most likely because suitable conditions for the vector are absent. Flavivirus exposure was detected in only one patient living in Sukkur. This may be a function of vector biology or the low number of screened patients. Sukkur is a small, sparsely populated district where the climate is very hot, windy, and dry, which is not suited to most mosquitoes.

Commercial assays make field work in limited areas feasible. They are easy to use and results are easy to interpret. However, especially for WNV, the assay is expensive. As expected, there was significant cross-reactivity between WNV and JEV when using IgM ELISA kits. If JEV and WNV ELISA data are grouped as "flavivirus exposed," roughly 13% of screened patients were positive. Consistent with the algorithm, all of the WNV and JEV samples will be tested by plaque reduction neutralization test (PRNT) to confirm which disease agent was present.

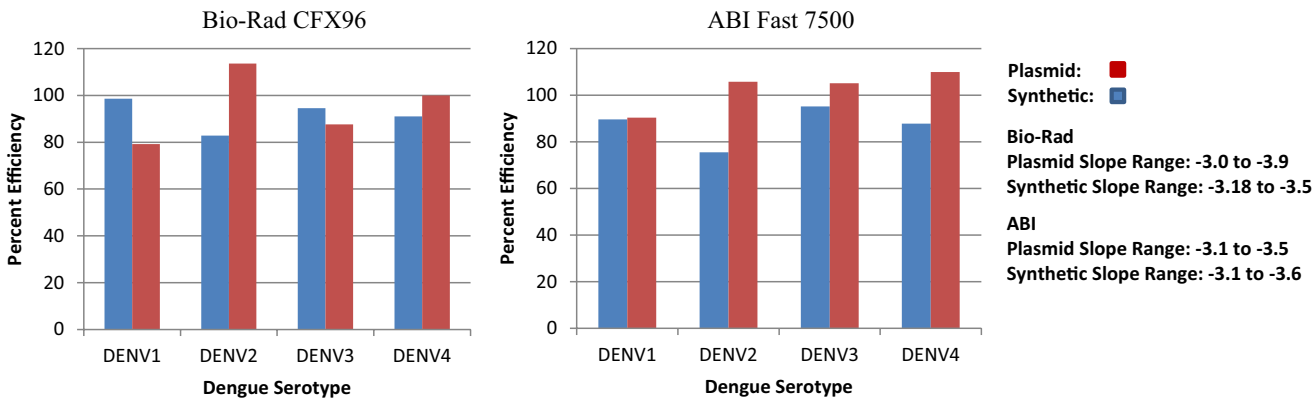

FIGURE 3 | RT-PCR efficiencies of synthetic DNA targets of dengue virus compared to the RT-PCR efficiencies of conventional dengue virus DNA from plasmids.

This cooperative BEP with Pakistan shows that scientific and medical projects can be successful and rapidly established between academic professional of countries that have limited political relations. Relationships were enhanced through early discussions between partners that included cultural and scientific barriers to health to optimize scientific training and develop methods for clinical surveillance. Barriers to recruitment of patients included lack of understanding of basic clinical signs of arboviral diseases on the part of health-care professionals. This was remedied *via* focused training study personnel who were employed locally.

Supply and communication lines also needed to be addressed by investment of research time into questions that challenge issues of data transfer and cold-chain supply without the need for development of entirely new technologies. For assay development, reagents and assays were developed to obviate the need for a cold-chain. In particular, it was determined that the BioRad chemistries could be shipped at ambient temperature and was confirmed by sending the reagents from the UF laboratory to the Aga Khan laboratory. When using plasmid technology, problems arose with reagent stability if shipping was interrupted (>3 weeks); thus, commercially manufactured real-time targets were used. These targets proved to be highly stable and exceptionally cost-effective. This also decreased the need to send supplies on dry ice that offered a substantial decrease in shipping costs and increased the availability of other forms of freight.

One of the most common challenges faced in the establishment of this project centered on limited communication to establish the needs of the US–Pakistan collaborators themselves. Most communications were written and did not include face-to-face fact finding before embarking on training and teaching sessions. In addition, problems with computer-based communication and travel (both local and abroad) delayed development of laboratory expertise. At the study sites, basic mobile phone technology was relied upon to share patient cases with infectious disease experts and the availability of a freely hosted website greatly facilitated this. Differences in compliance requirements at both University and government levels posed significant obstacles for transfer of medical technology.

Despite these issues, many goals have been attained within the first year of establishment of this project; a collaborative environment between a US-based University and a Pakistan-based University for the purposes of research and training exchange and a multisite network for arbovirus surveillance in humans across one of the largest and most populous provinces of Pakistan were established. US partners gained an understanding of the climatic, geographic, and cultural landscape of Pakistan and how this may contribute to arbovirus expansion. Finally, for the first time, preliminary assessment of the variety of several important arboviruses was determined indicating the need for continued surveillance and testing.

AUTHOR CONTRIBUTIONS

The following authors contributed to this manuscript in the following ways: contribution to the conception and design of the work: ML, EK, and KB; acquisition, analysis, and interpretation of data: ML, EK, DP, KB, AK, AN, JF, SS, FM, RH, and JL; drafting, editing, revising, and approving drafts: ML, KB, EK, DP, AK, AN, JF, SS, FM, RH, JL. ML, EK, and JL. All agreed to be accountable for all aspects of the work.

ACKNOWLEDGMENTS

We are grateful to Ms. Sally Beachboard, who has spent many hours determining supply routes for private vendors and in negotiating costs of supplies for our work in both the USA and Pakistan. We also thank Greg Gray for his initial work with Aga Khan University and epidemiological training.

FUNDING

This work was supported by the Defense Threat Reduction Agency, Basic Research Award # HDTRA1-14-1-0022, to the University of Florida. The contents do not necessarily reflect the position or the policy of the federal government, and no official endorsement should be inferred.

REFERENCES

1. Kitching P, Thomson G, Albrecht S, Sabara M, Werling D. Transboundary disease management: the theory and the practice – the science and the politics. *Transbound Emerg Dis* (2008) **55**:1–2. doi:10.1111/j.1865-1682.2007.01014.x
2. Leshner A, Turekian V. Harmonizing global science. *Science* (2009) **326**:1459–1459. doi:10.1126/science.1184624
3. Sharp PA, Leshner AI. Meeting global challenges. *Science* (2014) **343**:579. doi:10.1126/science.1250725
4. Campbell A, Restrepo C, Mackay D, Sherman R, Varma A, Ayala R, et al. Scalable, sustainable cost-effective surgical care: a model for safety and quality in the developing world, part I: challenge and commitment. *J Craniofac Surg* (2014) **25**:1674–9. doi:10.1097/SCS.0000000000001209
5. Corning S. World organisation for animal health: strengthening veterinary services for effective One Health collaboration. *Rev Sci Tech* (2014) **33**:639–50.
6. Ruano A, Friedman E, Hill P. Health, equity and the post-2015 agenda: raising the voices of marginalized communities. *Int J Equity Health* (2014) **13** 82–4. doi:10.1186/s12939-014-0082-6
7. Vandersmissen A, Welburn S. Current initiatives in one health: consolidating the one health global network. *Rev Sci Tech* (2014) **33**:421–32.
8. Fonkwo PN. Pricing infectious disease. The economic and health implications of infectious diseases. *EMBO Rep* (2008) **9**(Suppl 1):S13–7. doi:10.1038/embor.2008.110
9. De Paula SO, Fonseca BA. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *Braz J Infect Dis* (2004) **8**:390–8. doi:10.1590/S1413-86702004000600002
10. Rust RS. Human arboviral encephalitis. *Semin Pediatr Neurol* (2012) **19**:130–51. doi:10.1016/j.spen.2012.03.002
11. Paul RE, Patel AY, Mirza S, Fisher-Hoch SP, Luby SP. Expansion of epidemic dengue viral infections to Pakistan. *Int J Infect Dis* (1998) **2**:197–201. doi:10.1016/S1201-9712(98)90052-2
12. Khan E, Kisat M, Khan N, Nasir A, Ayub S, Hasan R. Demographic and clinical features of dengue fever in Pakistan from 2003-2007: a retrospective cross-sectional study. *PLoS One* (2010) **5**:e12505. doi:10.1371/journal.pone.0012505
13. Khan E, Siddiqui J, Shakoor S, Mehraj V, Jamil B, Hasan R. Dengue outbreak in Karachi, Pakistan, 2006: experience at a tertiary care center. *Trans R Soc Trop Med Hyg* (2007) **101**:1114–9. doi:10.1016/j.trstmh.2007.06.016
14. Khan E, Hasan R, Mehraj V, Nasir A, Siddiqui J, Hewson R. Co-circulations of two genotypes of dengue virus in 2006 out-break of dengue hemorrhagic fever in Karachi, Pakistan. *J Clin Virol* (2008) **43**:176–9. doi:10.1016/j.jcv.2008.06.003
15. Koo C, Nasir A, Hapuarachchi HC, Lee KS, Hasan Z, Ng LC, et al. Evolution and heterogeneity of multiple serotypes of dengue virus in Pakistan, 2006-2011. *Virol J* (2013) **10**:275. doi:10.1186/1743-422X-10-275
16. Fatima Z, Idrees M, Bajwa MA, Tahir Z, Ullah O, Zia MQ, et al. Serotype and genotype analysis of dengue virus by sequencing followed by phylogenetic analysis using samples from three mini outbreaks-2007–2009 in Pakistan. *BMC Microbiol* (2011) **11**:200–7. doi: 10.1186/1471-2180-11-200
17. Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, et al. Estimated global incidence of Japanese encephalitis: a systematic review. *B World Health Organ* (2011) **89**:766–74. doi: 10.2471/BLT.10.085233
18. Darwish MA, Hoogstraal H, Roberts TJ, Ahmed IP, Omar F. A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. *Trans R Soc Trop Med Hyg* (1983) **77**:442–5. doi:10.1016/0035-9203(83)90108-6
19. Mackenzie J. Emerging zoonotic encephalitis viruses: lessons from Southeast Asia and Oceania. *J Neurovirol* (2005) **11**:434–40. doi:10.1080/13550280591002487
20. Zohaib A, Saqib M, Beck C, Hussain M, Lowenski S, Lecollinet S, et al. High prevalence of West Nile virus in equines from the two provinces of Pakistan. *Epidemiol Infect* (2015) **143**:1931–5. doi:10.1017/S0950268814002878
21. Bryan JP, Iqbal M, Ksiazek TG, Ahmed A, Duncan JF, Awan B, et al. Prevalence of sand fly fever, West Nile, Crimean-Congo hemorrhagic fever, and leptospirosis antibodies in Pakistani military personnel. *Mil Med* (1996) **161**:149–53.
22. Sugamata M. Dependence on the birth season of the antibody level against West Nile virus in the Pakistani population. *Acta Virol* (1988) **32**:138–47.
23. Sugamata M, Ahmed A, Miura T, Takasu T, Kono R, Ogata T, et al. Seroepidemiological study of infection with West Nile virus in Karachi, Pakistan, in 1983 and 1985. *J Med Virol* (1988) **26**:243–7. doi:10.1002/jmv.1890260304
24. Igarashi A, Tanaka M, Morita K, Takasu T, Ahmed A, Akram DS, et al. Detection of west Nile and Japanese encephalitis viral genome sequences in cerebrospinal fluid from acute encephalitis cases in Karachi, Pakistan. *Microbiol Immunol* (1994) **38**:827–30. doi:10.1111/j.1348-0421.1994.tb01866.x
25. Nujum ZT, Thomas A, Vijayakumar K, Nair RR, Pillai MR, Indu PS, et al. Comparative performance of the probable case definitions of dengue by WHO (2009) and the WHO-SEAR expert group (2011). *Pathog Glob Health* (2014) **108**:103–10. doi:10.1179/2047773214Y.0000000131
26. Santiago GA, Vergne E, Quiles Y, Cosme J, Vazquez J, Medina JF, et al. Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. *PLoS Negl Trop Dis* (2013) **7**:e2311–26. doi:10.1371/journal.pntd.0002311
27. Solomon T, Vaughn DW. Pathogenesis and clinical features of Japanese encephalitis and West Nile virus infections. *Curr Top Microbiol Immunol* (2002) **267**:171–94. doi:10.1007/978-3-642-59403-8_9
28. Murray K, Baraniuk S, Resnick M, Arafat R, Kilborn C, Cain K, et al. Risk factors for encephalitis and death from West Nile virus infection. *Epidemiol Infect* (2006) **134**:1325–32. doi:10.1017/S0950268806006339

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 November 2015
doi: 10.3389/fmicb.2015.01128

Biosurveillance of avian influenza and Newcastle disease viruses in the Barda region of Azerbaijan using real time RT-PCR and hemagglutination inhibition

Shalala Zeynalova[1], Fizuli Guliyev[1], Mahira Vatani[1] and Bahruz Abbasov[2]*

[1] Virology Department, Republican Veterinary Laboratory, Baku, Azerbaijan, [2] Virology Department, Barda Zonal Veterinary Laboratory, Baku, Azerbaijan

The Azerbaijan State Veterinary Control Service (SVCS) has conducted active serological surveillance for avian influenza (AI) in poultry since 2006, when the first outbreak of AI H5N1 occurred in Azerbaijan. Samples are collected from September to May annually and tested using a hemagglutination inhibition (HI) assay to detect antibodies against H5 AI viruses. HI testing is also performed for Newcastle disease virus (NDV) upon request, but since this method cannot distinguish between natural infections and immune responses to vaccination, all positive results require follow-up epidemiological investigations. Furthermore, blood collection for the surveillance program is time-intensive and can be stressful to birds. In order to improve the national surveillance program, alternative sampling and testing methodologies were applied among a population of birds in the Barda region and compared with results of the national surveillance program. Tracheal and cloacal swabs were collected instead of blood. Rather than testing individual samples, RNA was pooled to conserve resources and time, and pools were tested by real-time reverse transcription polymerase chain reaction (rRT-PCR). Environmental sampling at a live bird market was also introduced as another surveillance mechanism. A total of 1,030 swabs were collected, comprising tracheal, and cloacal samples from 441 birds and 148 environmental surface samples from farms or the live bird market. During the same time, 3,890 blood samples were collected nationally for the surveillance program; 400 of these samples originated in the Barda region. Birds sampled for rRT-PCR were likely different than those tested as part of national surveillance. All swab samples tested negative by rRT-PCR for both AI and NDV. All blood samples tested negative for H5 by HI, while 6.2% of all samples and 5% of the Barda samples tested positive for exposure to NDV. Follow-up investigations found that positive samples were from birds vaccinated in the previous month. This study demonstrated that taking swabs was quicker and less invasive than blood collection. Results of rRT-PCR testing were similar to HI testing for H5 but also ruled out infection with all influenza type A viruses and not just H5. In addition, rRT-PCR testing was able to rule out active infections with NDV.

Keywords: avian influenza, Newcastle disease, Azerbaijan, environmental surveillance, live bird market

OPEN ACCESS

Edited by:
Nathan Wolfe, Metabiota, USA

Reviewed by:
Sherry Layton, Vetanco, Argentina
Mamoona Chaudhry, University of Veterinary and Animal Sciences, Lahore, Pakistan

****Correspondence:***
Shalala Zeynalova
zeynalova.shelale@rambler.ru

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *19 June 2015*
Accepted: *28 September 2015*
Published: *06 November 2015*

Citation:
Zeynalova S, Guliyev F, Vatani M and Abbasov B (2015) Biosurveillance of avian influenza and Newcastle disease viruses in the Barda region of Azerbaijan using real time RT-PCR and hemagglutination inhibition. Front. Microbiol. 6:1128. doi: 10.3389/fmicb.2015.01128

INTRODUCTION

Influenza A virus subtype H5N1, also known as highly pathogenic avian influenza (HPAI) or simply H5N1, is a virus that infects birds and can cause serious illness and death in humans (Liu et al., 2005; Alexander and Capua, 2009; Belser et al., 2011). The first case of human H5N1 was recorded in 1997 during a poultry outbreak in Hong Kong. After its re-emergence in 2003 and 2004, the virus spread from Asia to Europe and Africa, where it has since resulted in over 800 human infections and 400 deaths (World Health Organization [WHO], 2005; Gilsdorf et al., 2006; Belser et al., 2011). Migratory birds can spread H5N1 and other avian viruses over long distances (OIE World Organization for Animal Health, 2012). AI outbreaks in poultry have a negative impact on livelihoods, local economies, and international trade in affected countries. The circulation of H5N1 in poultry also threatens public health, since this strain has the potential to cause serious disease in people and is capable of mutating into a form that is more transmissible among humans, increasing the risk of a pandemic and mass mortality event.

Influenza virus subtypes other than H5N1 that circulate in poultry and other animals also pose a threat to public health. For example, influenza A viruses of the H7 hemagglutinin type can be highly pathogenic in birds and must be reported to the OIE World Organization for Animal Health (2012) when they are detected. H7 AI viruses are also known to infect humans. A novel H7N9 virus emerged in 2013 that has caused a total of 571 laboratory-confirmed infections and 212 deaths to date as of February 23, 2015 (World Health Organization [WHO], 2015). H9 viruses also can cause infections in birds and people (Lin et al., 2000; Iqbal et al., 2009; Poovorawan et al., 2013). An H9N2 virus identified in poultry has been found to be responsible for contributing six genes to the H7N9 influenza virus that emerged in 2013 and is known to be pathogenic in people (Pu et al., 2014).

Newcastle disease (ND) is a highly contagious viral avian disease that is a differential diagnosis for HPAI as it causes clinical symptoms similar to those of HPAI, including damage to the respiratory, intestinal, and nervous systems of infected birds. ND has been classified by the OIE as an especially dangerous disease (Group A) on account of its ability to adversely affect the health of large populations of birds. Newcastle disease virus (NDV), the causative agent of ND, is known to infect over 200 species of birds (World Organization for Animal Health, 2008). The severity of disease varies by host species and strain of virus; the fatality rate in birds infected with NDV has reached 100% in past outbreaks (Agayeva and Zeynalova, 2011). In recent years, the reported incidence of ND has increased worldwide; outbreaks have been detected in Europe, America, Asia, and Africa (Gilsdorf et al., 2006).

Azerbaijan has recorded outbreaks of both viruses in poultry, most recently in 2006. In February 2006, H5N1 AI was first identified in Azerbaijan among wild birds along the coast of the Caspian Sea near Baku. Later that month, infections were detected in poultry on farms in the northeastern and southern regions of the country. The first cases of human H5N1 infection were recorded in March, and by December 2006 the disease had spread throughout Azerbaijan, resulting in eight confirmed human cases, five of which were fatal. The outbreaks were ultimately traced to wild birds and domestic poultry in the Gilazi and Bilasuvar rayons (Agayeva and Zeynalova, 2011; Belser et al., 2011). Two outbreaks of NDV were also recorded in 2006 (Agayeva and Zeynalova, 2011).

Following the 2006 outbreak of H5N1, the Azerbaijan State Veterinary Control Service (SVCS) initiated active serological monitoring for AI in poultry. Azerbaijan has a total of approximately 17 million poultry, of which about 70% are privately owned and 30% are commercially owned. The majority of poultry are considered village or backyard poultry as categorized by the FAO definition of poultry production systems (Food and Agriculture Organization [FAO], 2004). Under the SVCS program, blood samples from poultry are tested for the presence of antibodies against H5 by the hemagglutination inhibition (HI) assay using OIE-standardized reagents. Twelve Zonal Veterinary Laboratories (ZVLs) and Regional Veterinary Offices in Azerbaijan are engaged in this surveillance program. Sample collection from domestic birds occurs between September and May each year and testing is performed at the Republican Veterinary Laboratory (RVL). When the Regional Veterinary Offices submit samples for H5 testing, they may also request ND disease testing, which is performed by HI using OIE-standardized reagents. ND testing is requested for approximately half of the samples submitted.

The study described herein was undertaken to investigate sampling and testing methodologies that might be better alternatives to those currently deployed for AI and ND serosurveillance in Azerbaijan. HI testing of HPAI is subtype-specific, but the current surveillance system only tests for H5 viruses (State Veterinary Control Service, 2013), and therefore will fail to detect immune responses to other subtypes (including known human-pathogenic strains such as H7 and H9). In addition, HI testing requires blood samples, which are time-intensive and more invasive to collect than other types of biological samples (such as tracheal and cloacal swabs).

The specific goals of this study were to use rRT-PCR to test tracheal and cloacal swab samples for AI and ND viral RNA; to screen samples for the influenza A matrix gene so as to detect all subtypes known to be pathogenic to humans; to implement sample pooling as a way to reduce the costs of testing; and to introduce environmental sampling in live bird markets as a way to increase the chances of detecting disease in an area where species of birds from many locations are commingled. The OIE Terrestrial Manual states that while virus culture is the "gold standard" diagnostic test for identifying virus, culture is laborious and time insensitive. Real-time RT-PCR is the diagnostic method of choice in many laboratories for identifying the presence of antigen (OIE World Organization for Animal Health, 2012). Several studies have shown the rRT-PCR has been shown to have the added benefit of being able to pool samples as a way to save money on reagents for both AI and ND testing (Fereidouni et al., 2012; Spackman et al., 2013). Live bird markets have long been known to be a source of influenza and NDVs. Influenza A H5, H7, and H9 viruses, which can affect both poultry and people, have all been identified in poultry or the environment of live bird markets (Liu et al., 2003; Ge et al., 2009; Wan et al., 2011; Lee

et al., 2013; Waziri et al., 2014). NDV has also been identified in live bird markets (Jibril et al., 2014; Mulisa et al., 2014). Environmental sampling of live bird markets has been shown to be an effective way to assess influenza contamination (Indriani et al., 2010). In one study, PCR of environmental samples was a more effective method for identifying the presence of influenza virus contamination than viral culture (Horm et al., 2013).

In order to investigate sampling and testing methodologies that might be better alternatives or supplements to those methods currently deployed for AI and ND serosurveillance in Azerbaijan, domestic poultry were sampled between September 2013 and April 2014 using new methodologies aimed at detecting the presence of antigen. The results of testing were compared with results of the ongoing national surveillance aimed at detecting immune response against AI A H5 viruses.

MATERIALS AND METHODS

Site Selection

Project activities were centered in Barda, a region with a documented history of H5N1 and ND cases in birds (Agayeva and Zeynalova, 2011). The region is regularly exposed to migratory birds because it is situated between the Mingechevir water reservoir to the north and the Goygol and Agh Gol National Parks to the west and the southeast, respectively. According to the Azerbaijan Ministry of Ecology and Natural Resources, these areas comprise part of the migratory flyway for several wild bird species. Moreover, Barda city hosts the largest live animal market in Azerbaijan. Finally, of all the rayons participating in the SVCS surveillance program for AI, Barda historically generates the most samples and routinely requests the most ND testing.

Project investigators conducted an initial sample collection trip in May 2013, followed by eight more collection trips between September 2013 and April 2014. Samples were collected at the live bird market in Barda city during each of the nine collection trips. In addition, samples were collected from farms or households in 17 of 110 villages and Barda city. To randomly select villages for sampling, every village in the Barda rayon was assigned a number, and numbers were randomly selected from this list using the RAND function in Microsoft Excel. To identify farms for sampling, investigators would randomly choose one of four directions from the center of each selected village by spinning a bottle from the center and choosing the direction that the bottle pointed in. Every other farm or household was selected in the chosen direction, up to a total of three farms or households with poultry. If fewer than three farms or households existed in a given direction within the boundaries of a village, another direction was chosen randomly to continue the study. The geo-coordinates (latitude and longitude) of the farms/households and market where sampling occurred were captured using an eTrex10 GPS unit (Garmin, Olathe, KS, USA) and recorded in an Excel spreadsheet.

Sample Collection

Samples for testing by rRT-PCR were collected from domestic birds (ducks, geese, turkeys, and chickens) found at the selected farms/households from Sector 4 production systems (village or backyard poultry with minimal biosecurity and birds consumed locally) as categorized by the Food and Agriculture Organization [FAO] (2004). At the live bird market, the collection teams solicited the voluntary participation of poultry vendors during six site visits. Cloacal and tracheal samples were collected from each of the birds enrolled in this study (**Figure 1**). Cloacal sampling was performed by gently inserting a sterile cotton-tipped swab (Puritan, Inc., Guilford, ME, USA) approximately 1 cm into the vent. The tip of the swab was rolled along the interior surface of the cloaca before it was removed and placed into a sterile conical tube for transport to the laboratory. To collect tracheal samples, a sterile cotton-tipped swab was inserted as deeply as practicable into the oropharynx of the bird being tested. The investigators made a special effort to bring the sterile swab in contact with the trachea and not simply the back of the oral cavity. The swab was withdrawn and placed into a sterile conical tube without media for transport to the laboratory. During the collection procedures, the birds were restrained manually without sedation, anesthesia, or other medications. Sampling activities were conducted according to SVCS guidelines which are the same as the (Food and Agriculture Organization [FAO], 2006) guidelines and no birds were injured during collection.

Environmental swabs were collected in areas where birds were held while awaiting sale at the live bird market. A dry, sterile, cotton-tipped swab without media was used to swab cages, tables, and wooden boards contaminated with the excretions of caged birds. Each sample, regardless of its source, was placed into an individual sterile conical tube to avoid any cross-contamination that could confound the results of the diagnostic testing. All samples were transported to the Barda ZVL on ice on the day of collection, where they were stored at –20°C pending transport to the RVL in Baku for testing at the end of the sampling period.

Sample size was calculated to estimate the prevalence of AI in poultry in the Barda region considering all poultry as one population due to the minimal biosecurity in place for backyard farms and the live poultry market. As the actual prevalence of

FIGURE 1 | Collecting swab samples from birds at Kelenterli village.

AI or ND in this population was not known, the investigators assumed a prevalence of 50% to conservatively determine the appropriate sample size. From this assumption, the minimum number of birds to sample was estimated to be 385 (with a 95% confidence interval and 5% allowable error). Sample size was calculated using WinEpiscope 2.0 (Thrusfield et al., 2001). All birds available at a selected farm were swabbed, which on average was approximately four birds per farm.

This project was conducted with approval by the Azerbaijan State Veterinary Control Service (SVCS) Scientific Committee, which reviewed the project proposal for scientific and ethical concerns, including the use of animals. This work was funded through a grant from the Cooperative Biological Engagement Program (CBEP); the Medical Research and Materiel Command (MRMC) Animal Care and Use Review Office (ACURO) determined that the protocols described surveillance methods, and therefore Institutional Animal Care and Use Committee (IACUC) oversight was unnecessary. Additionally, bird sampling procedures were deemed to cause less stress to the birds than those normally conducted as part of the national surveillance for H5 AI viruses. No birds were harmed during any part of the sampling associated with this project. All sample collection activities were completed in accordance with appropriate biosafety procedures. Participants donned appropriate personal protective equipment for the collection process, including disposable gowns, gloves, hair nets, goggles, N95 respirators, and shoe covers.

Sample Management and Preparation

Each sample was systematically assigned a unique identification number at the time of collection to facilitate matching of tracheal and cloacal swabs collected from the same bird. A record sheet was created to track the samples. The information captured on the specimen records included the sample identification number, species sampled (turkey, ducks, geese, chickens), and sample origin (i.e., cloaca, trachea, or environmental surface). If the bird had any outward clinical signs of illness such as discharge or diarrhea, this was also noted. The samples were shipped on ice to the RVL at the end of each monthly collection trip; total transit time between Barda and Baku is approximately 4 h. Upon arrival at the RVL, the samples were stored at –20°C pending RNA extraction, which typically occurred within 1 week of sample arrival. Each sample was extracted individually and then pooled for viral RNA testing with rRT-PCR.

Real-time RT-PCR Analysis

All 1,030 samples collected for rRT-PCR were extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. After the RNA was extracted from individual samples, a portion of the viral RNA from up to five samples of the same epidemiological unit and sample type was pooled. A total of 213 pools were created from the samples collected over the course of the project.

Real-time RT-PCR amplification was performed using the SuperScript II One-Step RT-PCR System (Invitrogen Inc., Carlsbad, CA, USA); primer/probe sets (Sigma–Aldrich Inc., Germany); and a Lightcycler 2.0 instrument (Roche Diagnostics, Indianapolis, IN, USA). Invitrogen Platinum Taq DNA polymerase kits (Life Technologies, Carlsbad, CA, USA) were used to prepare the master mix for each pathogen (influenza A virus or NDV). Real-time RT-PCR testing for a conserved region of the influenza A matrix gene was used to screen samples generically for all influenza A virus (AIV) strains (Spackman et al., 2002). Real-time RT-PCR testing for the Fusion gene was conducted to detect NDV as previously described (Wise et al., 2004; Alexander and Capua, 2009). The specific forward and reverse primers used for testing both AIV and NDV are shown in **Table 1**. A total of 4 μL of extracted pooled RNA was used in each 20 μL reaction. Positive and negative controls were included in each run and each sample was run in duplicate. Had any samples tested positive for the AIV matrix gene, H5, H7, and/or H9 subtype-specific primers and probes would have been used to characterize the virus subtype.

National Active Surveillance and Vaccination Program for AI H5 and ND

Since 2006, the Azerbaijan SVCS has conducted an annual serosurveillance program in which all 12 regional ZVLs participate (**Table 2**). Each ZVL is responsible for collecting samples from 4 to 5 rayons to ensure that surveillance occurs throughout the country. Field veterinarians collect serum samples from domestic fowl, and tissue samples are collected from dead wild birds in Azerbaijan's national parks and nature reserves. Veterinarians submit collected samples to the local ZVL, which in turn sends them to RVL for testing. Testing for AI H5

TABLE 1 | Primer/probe sequences and target pathogens and genomes.

Primer/Probe	Sequence*	Pathogen target	Genome target
Influenza A virus			
AI gene M forward primer	5′ AGATGAGTCTTCTAACCGAGGTCG 3′	AIV	Matrix
AI gene M reverse primer	5′ TGCAAAAACATCTTCAAGTCTCTG 3′		
Probe AI gene M	FAM-TCAGGCCCCCTCAAAGCCGA –TAMRA		
Newcastle disease virus			
Forward primer	5′ GGTGAGTCTATCCGGARGATACAAG 3′	NDV	Fusion
Reverse primer	5′ AGCTGTTGCAACCCCAAG 3′		
Probe	5′ [FAM]AAGCGTTTCTGTCTCCTTCCTCCA [BHQ] 3′		

**Fluorescent dye abbreviations: 6FAM, 6-Carboxyfluorescein; TAMRA, tetramethylrhodamine; BHQ1 = Black Hole Quencher® -1.*

TABLE 2 | Total number of samples tested for avian influenza and Newcastle disease viruses during active surveillance from 2011 to 2014.

Year	Domestic poultry blood tested for H5	Wild bird internal organs tested for H5	Domestic poultry blood tested for NDV
2011	8,752	110	4,020
2012	6,192	79	3,000
2013	7,353	18	3,700
2014	7,074	127	4,000

(and ND, when requested) is conducted by the SVCS according to standard HI protocols (Alexander and Capua, 2009; OIE World Organization for Animal Health, 2012).

The SVCS conducts an annual vaccination program against ND using two live lentogenic virus vaccines, including H and LaSota vaccines. No vaccination is conducted for AI.

RESULTS

Sample Collection

Overall, 1,030 swab samples were collected from birds and the environment for testing by rRT-PCR over nine sampling events (one event each in May, September, October, November, and December 2013, and January and April 2014; two events in March 2014). A total of 441 birds were sampled including chickens ($n = 341$), turkeys ($n = 54$), geese ($n = 24$), and ducks ($n = 22$). Of the 441 birds sampled, 89 were from the live market while 352 were from private farms in the 17 villages and the city of Barda (**Figure 2**). Of 441 birds sampled, 301 (68%) of these birds had been previously vaccinated against ND with either the LaSota or the H vaccine. One bird exhibited nasal discharge at the time of sampling; all other birds were clinically healthy. The investigators also collected 134 environmental swabs from the live bird market and 14 environmental samples from farms (**Table 3**).

Real-time RT-PCR Analysis

A total of 213 RNA pools were tested for NDV and for the influenza A virus matrix gene by rRT-PCR. None of the pooled samples were positive for either of the agents (**Table 4**). No specific subtype testing for H5, H7, or H9 was necessary.

National Active Surveillance and Vaccination Program for AI H5 and ND

Between September 2013 and May 2014, the national surveillance program collected a total of 3,890 samples for AI virus testing throughout Azerbaijan; 400 of these originated from domestic birds in the Barda region, but were likely different than those collected for rRT-PCR testing. None of the 3,890 samples were positive for H5 AIV by the HI assay. A total of 240 samples (6.2%) were positive for NDV overall; 20 (5%) of the 400 samples from Barda were positive. According to 2014 SVCS data, 19,631,361 domestic birds were inoculated against NDV with the H vaccine and 18,639,580 birds were vaccinated with the LaSota vaccine.

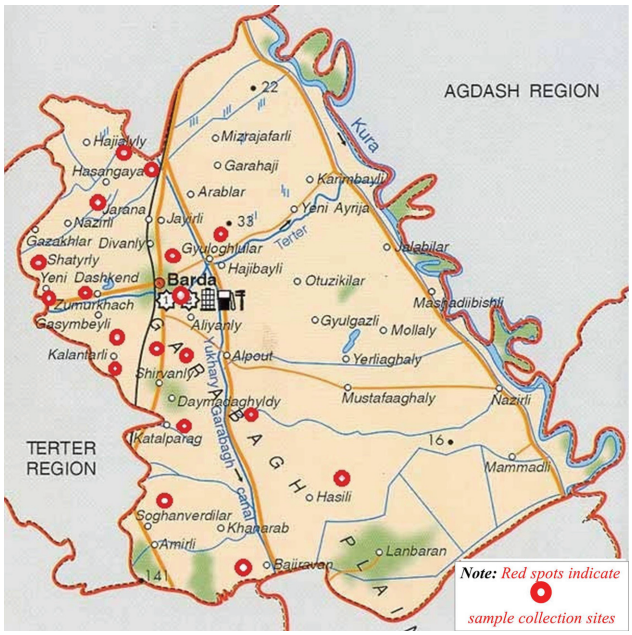

FIGURE 2 | Red circles indicate sampling sites in the Barda region of Azerbaijan.

DISCUSSION

The results from the rRT-PCR testing conducted in this study aligned with the results of the SVCS HI testing program for H5, which has not detected any birds infected with AI H5 since 2006. The rRT-PCR test used in this study targeted the influenza matrix A gene. Because this gene is common to all influenza A viruses, it can be used to screen for several different strains of influenza type A viruses. A positive result on the rRT-PCR assay for the matrix gene would be an indication for additional testing to identify the specific influenza strain present in the sample. The AI HI assay only detects AI H5 and does not test for the presence of other hemagglutinin types. Although the results appear to be consistent, a direct comparison between the results was not possible because the samples collected in the national surveillance program and the Barda study were not from the same birds. Regardless, the failure of two different testing strategies to detect H5 suggests that this strain of AIV is not present in the Barda region.

This study showed that real-time rRT-PCR testing could be a valuable addition to the national surveillance program. Among the samples from Barda that SVCS tested for NDV, 5% had detectable antibodies. The detection of antibodies indicates that the birds were exposed to NDV antigen either through natural infection or exposure to a vaccine that triggered an immune response detectable by serology testing. The SVCS vaccinates birds annually with live lentogenic virus vaccines, and a positive serology test result would not be unexpected. No large-scale die-offs or symptoms consistent with NDV were reported in Azerbaijan during the surveillance period. Coupled with the lack of positive rRT-PCR test results, this indicates that none of the

TABLE 3 | Number and type of samples collected from the Barda region (2013–2014).

Collection period	Location	# of farms	# of samples collected				
			Trachea	Cloaca	Environ	Samples per location	Total
23–24 May 2013	Live bird market	N/A	15	15	0	30	104
	Households in central Barda	N/A	15	15	2	32	
	Zumurkhan village	5	20	20	2	42	
26–27 September 2013	Live bird market	N/A	10	10	0	20	108
	Imamgulubeyli village	5	15	15	4	34	
	Chemenli village	8	26	26	2	54	
23–25 October 2013	Live bird market	N/A	18	18	7	43	163
	Qara-Yusifli village	10	40	40	0	80	
	Bala-Qecher village	10	20	20	0	40	
13–15 November 2013	Live bird market	N/A	10	10	9	29	153
	Qahramanli village	9	30	30	2	62	
	Shatirli village	10	30	30	2	62	
18–20 December 2013	Live bird market	N/A	16	16	8	40	100
	Garana village	3	15	15	0	30	
	Guloglular village	5	15	15	0	30	
27–29 January 2014	Live bird market	N/A	20	20	8	48	90
	Seyif Yusifli village	2	10	10	0	20	
	Hadjali village	3	11	11	0	22	
14 March 2014	Live bird market	N/A	0	0	30	30	30
16–19 March 2014	Ketelparaq village	3	18	18	0	36	252
	Soganverdiler village	3	18	18	0	36	
	Muganli village	3	18	18	0	36	
	Kelenterli village	3	17	17	0	34	
	Qasimbeyli village	3	17	17	0	34	
	Alakadirli village	3	17	17	0	34	
	Live bird market	N/A	0	0	42	42	
19 April 2014	Live bird market	N/A	0	0	30	30	30
Total		88	441	441	148	1,030	1,030

birds tested were actively infected. In the absence of clinical evidence of a ND outbreak, it is likely that birds were exposed to NDV antigen through vaccination rather than natural infection. Further epizootological investigation conducted by the district veterinarian concluded that the positive results detected were likely the result of the vaccine administered a month before sampling. Additional studies comparing the results of rRT-PCR and serological assays on samples collected from the same birds would help to establish rRT-PCR testing as a viable addition to conventional serology testing to rule out active infections, or as an alternative testing method to minimize false positive test results.

TABLE 4 | rRT-PCR screening results of pooled samples by collection month.

Sampling month and year	Number of Pools	Newcastle disease results	Influenza A matrix gene results
May 2013	34	Negative	Negative
September 2013	22	Negative	Negative
October 2013	33	Negative	Negative
November 2013	29	Negative	Negative
December 2013	20	Negative	Negative
January 2014	20	Negative	Negative
March 2014	50	Negative	Negative
April 2014	5	Negative	Negative
Total	213		

Other studies have similarly applied both HI and rRT-PCR to surveillance systems, using HI to detect seropositivity and using rRT-PCR to detect active infections. A surveillance program for H5 and H7 AIV in Poland used RT-PCR to investigate active infections following a positive HI result (Pikula et al., 2014). Out of 45,000 serum samples, nine geese or ducks were positive for H5 or H7 on HI. When tested by RT-PCR, all were negative. The conclusion of the authors was that a low pathogenic AIV had previously infected some birds, but that there was no actively circulating viral infections (Pikula et al., 2014). Another surveillance program aimed at evaluating AIVs in waterfowl in Spain identified 1.1 and 0.3% of birds seropositive for H5 and H7. Of 47 samples that tested positive by both HI and ELISA, all tested negative by rRT-PCR (Jurado-Tarifa et al., 2014). A study using both HI and RT-PCR to investigate ND in caged birds in Tehran, found that RT-PCR was more sensitive for detection of active carriers than was the HI test when 35 of 335 tested positive by RT-PCR but were not positive by HI (Madadgar et al., 2013).

There are several potential explanations for the lack of any positive rRT-PCR results. First, there may be no AIV or NDV circulating in the study area; this possibility is supported by the absence of clinical evidence of disease caused by either virus. Most birds in this study appeared healthy; only one farm had one sick bird which tested negative for both AIV and NDV. Alternatively, flaws in the study design and execution may have interfered with detection of positive samples, despite efforts to maximize the success of the project. For example, the villages selected for the study may not have been conducive to disease detection due to previous vaccination efforts against NDV. Excluding vaccinated birds from studies is problematic because the vaccination status of individual birds may be difficult to ascertain. In a real-world setting, such as the live bird market or a village farm, the owner of a given bird may not be fully aware of its provenance, especially when live birds are bought and sold in an uncontrolled market. In the absence of a controlled study, future investigations could consider two alternative research directions. One approach would be to collect samples in isolated regions where no vaccinated birds are known to exist. Alternatively, samples for rRT-PCR testing and for serology testing could be collected from the same bird. Birds that are positive by serology tests but negative by rRT-PCR are likely to have been vaccinated; birds that are positive by both assays are most likely infected, either because they were not vaccinated or because the vaccine was ineffective.

This project introduced environmental testing as a new method to detect viral diseases that may be present at live bird markets. Such environments can present an opportunity for continued viral transmission as healthy birds come into contact with birds that may be infected. Environmental testing is not invasive, so it is more likely to be accepted by vendors concerned about the adverse effects that sampling could have on their birds. Environmental sampling is also safer for veterinary health officers, since it avoids the need to directly handle and sample live birds. Although the utility of environmental testing could not be verified in this study due to the lack of positive results, this approach has been validated as a method for virus detection in similar studies (Indriani et al., 2010).

This study demonstrated that swabs are a safe and easy way to collect samples from live birds, and that environmental sampling at live bird markets merits further consideration for inclusion into the Azerbaijani surveillance program. Real-time RT-PCR testing should also be considered as an addition or substitution to the program, because it can be used to identify all influenza type A viruses (including H7 and H9 viruses known to be pathogenic to humans) and can help to rule out active infection of NDV in birds testing falsely positive as a result of vaccination.

ACKNOWLEDGMENTS

The authors are grateful to Cooperative Biological Engagement Program for generously providing the funding for this project. We also thank Giovanni Cattoli, Director of the Department of Research and Innovation, Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), for his assistance with reagents and technical support. The authors also are grateful to Lisa Strelow, April Johnson, Peter Flanagan, Elshad Rzayev, Saida Aliyeva, and Sonya Narodny for support with the project and manuscript development.

REFERENCES

Agayeva, E. M., and Zeynalova, S. K. (2011). *Disease of Avian Influenza and Newcastle in Azerbaijan (Epizootology)*, Vol. 2. Baku: Azerbaijan Agriculture Science, 116–11829.

Alexander, D., and Capua, I. (2009). *Avian Influenza and Newcastle Disease*. Milan: Springer-Verlag.

Belser, J. A., Zeng, H., Katz, J. M., and Tumpey, T. M. (2011). Infection with highly pathogenic H7 influenza viruses results in an attenuated proinflammatory cytokine and chemokine response early after infection. *J. Infect. Dis.* 203, 40–48. doi: 10.1093/infdis/jiq018

Fereidouni, S. R., Harder, T. C., Gaidet, N., Ziller, M., Hoffman, B., Hammoumi, S., et al. (2012). Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. *J. Virol. Methods* 186, 119–125. doi: 10.1016/j.jviromet.2012.08.002

Food and Agriculture Organization [FAO] (2004). *FAO Recommendations on the Prevention, Control and Eradication of Highly Pathogenic Avian Influenza (HPAI) in Asia*. Available at: http://web.oie.int/eng/AVIAN_INFLUENZA/FAO%20recommendations%20on%20HPAI.pdf

Food and Agriculture Organization [FAO] (2006). *Sick & Dead Wild Bird Disease Surveillance; Sample Collection Protocol with emphasis on Highly Pathogenic Avian Influenza May 2006*. Available at: http://www.fao.org/avianflu/conferences/rome_avian/documents/Sickdeadbirdsurveillancefinal.pdf

Ge, F., Zhou, J. P., Liu, J., Wang, J., Zhang, W. Z., Sheng, L. P., et al. (2009). Genetic evolution of H9 subtype influenza viruses from live poultry markets in Shanghai, China. *J. Clin. Microbiol.* 47, 3294–3300. doi: 10.1128/JCM.00355-09

Gilsdorf, A., Boxall, N., Gasimov, V., Agayev, I., Mammadzade, F., Ursu, P., et al. (2006). Two clusters of human infection with influenza/H5N1 virus in the Republic of Azerbaijan, February-March 2006. *Euro Surveill.* 11, 122–126.

Horm, S. V., Sorn, S., Allal, L., and Buchy, P. (2013). Influenza A (H5N1) virus surveillance at live poultry markets, Cambodia. *Emerg. Infect. Dis.* 19, 305–308. doi: 10.3201/eid1902.121201

Indriani, R., Samaan, G., Gultom, A., Loth, L., Indryani, S., Adjid, R., et al. (2010). Environmental sampling for avian influenza virus A (H5N1) in live-bird markets, Indonesia. *Emerg. Infect. Dis* 16, 1889–1895. doi: 10.3201/eid1612.100402

Iqbal, M., Yaqub, T., Reddy, K., and McCauley, J. W. (2009). Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. *PLoS ONE* 4:e5788. doi: 10.1371/journal.pone.0005788

Jibril, A. H., Umoh, J. U., Kabir, J., Saidu, L., Magaji, A. A., Bello, M. B., et al. (2014). Newcastle disease in local chickens of live bird markets and households in Zamfara State, Nigeria. *ISRN Epidemiol.* 2014, 513961.

Jurado-Tarifa, E., Napp, S., Gomez-Pacheco, J. M., Fernandez Morente, M., Jaen-Tellez, J. A., Arenas, A., et al. (2014). Surveillance of influenza viruses in waterfowl used as decoys in Andalusia, Spain. *PLoS ONE* 9:e98890. doi: 10.1371/journal.pone.0098890

Lee, S. S., Wong, N. S., and Leung, C. C. (2013). Exposure to avian influenza H7N9 in farms and wet markets. *Lancet* 381, 1815. doi: 10.1016/S0140-6736(13)60949-6

Lin, Y. P., Shaw, M., Gregory, V., Cameraon, K., Lim, W., Klimov, A., et al. (2000). Avian-to-human transmission of H9N2 subtype influenza A viruses: relationahip between H9N2 and H5N1 human isolates. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9654–9658. doi: 10.1073/pnas.160270697

Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X., et al. (2005). Highly pathogenic H5N1influenza virus infection in migratory birds. *Science* 309, 1206. doi: 10.1126/science.1115273

Liu, M., He, S., Walker, D., Zhou, N., Perez, D. R., Mo, B., et al. (2003). The influenza virus gene pool in a poultry market in South central china. *Virology* 305, 267–275. doi: 10.1006/viro.2002.1762

Madadgar, O., Karimi, V., Nazaktabar, A., Kazemimanesh, M., Ghafari, M. M., Azimi Dezfouli, S. M., et al. (2013). A study of Newcastle disease virus obtained from exotic caged birds in Tehran between 2009 and 2010. *Avian Pathol.* 42, 27–31. doi: 10.1080/03079457.2012.752791

Mulisa, D., W/Kiros, M. K., Alemu, R. B., Keno, M. S., Furaso, A., Heidari, A., et al. (2014). Characterization of newcastle disease virus and poultry-handling practices in live poultry markets, ethiopia. *Springerplus* 3, 459. doi: 10.1186/2193-1801-3-459

OIE World Organization for Animal Health (2012). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012*. Available at: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/

Pikula, A., Smietanka, K., Lisowska, A., and Minta, Z. (2014). Active surveillance in poultry in Poland for avian influenza subtypes H5 and H7. *Acta Biochim. Pol.* 61, 459–463.

Poovorawan, Y., Pyungporn, S., Prachayangprecha, S., and Makkoch, J. (2013). Global alert to avian influenza viruse infection: from H5N1 to H7N9. *Pathog. Glob. Health* 107, 217–223. doi: 10.1179/2047773213Y.0000000103

Pu, J., Wang, S., Yin, Y., Zhang, G., Carter, R. A., Wang, J., et al. (2014). Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. *Proc. Natl. Acad. Sci. U.S.A.* 112, 548–553. doi: 10.1073/pnas.1422456112

Spackman, E., Pedersen, J. C., McKinley, E. T., and Gelb, J. Jr. (2013). Optimal specimen collection and transport methods for he detection of avian influenza virus and Newcastle disease virus. *BMC Vet. Res.* 9:35. doi: 10.1186/1746-6148-9-35

Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., et al. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.* 40, 3256–3260. doi: 10.1128/JCM.40.9.3256-3260.2002

State Veterinary Control Service (2013). *State Veterinary Control Service*. Available at: http://vet.gov.az/az/content/category/23

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J. P., and Frankena, K. (2001). Win Episcope 2.0: improved epidemiological software for veterinary medicine. *Vet. Rec.* 148, 567–572. doi: 10.1136/vr.148.18.567

Wan, S. V., Dong, L., Lan, Y., Long, L. P., Xu, C., Zou, S., et al. (2011). Indications that live poultry markets are a major source of human H5N1 influenza virus infection in China. *J. Virol.* 85, 13432–13438. doi: 10.1128/JVI.05266-11

Waziri, N. E., Nguku, P., Olayinka, A., Ajayi, I., Kabir, J., Okolocha, E., et al. (2014). Evaluating a surveillance system: live-bird market surveillance for highly pathogenic avian influenza, a case study. *Pan Afr. Med. J.* 18(Suppl. 1), 11. doi: 10.11694/pamj.supp.2014.18.1.4188

Wise, M. G., Suarez, D. L., Seal, B. S., Pedersen, J. C., Senne, D. A., King, D. J., et al. (2004). Development of a real-time reverse transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *J. Clin. Microbiol.* 42, 329–338. doi: 10.1128/JCM.42.1.329-338.2004

World Health Organization [WHO] (2005). Consultation on human influenza A/H5 avian influenza A (H5N1) infection in humans: the writing committee of the World Health Organization (WHO) consultation on human influenza A/H5. *N. Engl. J. Med.* 353, 1374–1385. doi: 10.1056/NEJMra052211

World Health Organization [WHO] (2015). *Risk Assessment of Human Infections with Avian Influenza A (H7N9) Virus. 23 February 2015*. Available at: http://www.who.int/influenza/human_animal_interface/influenza_h7n9/RiskAssessment_H7N9_23Feb20115.pdf?ua=1 [accessed September 3, 2015].

World Organization for Animal Health (2008). *Newcastle Disease*, NEWC_A2008, 1–7.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 19 October 2015
doi: 10.3389/fpubh.2015.00235

International biological engagement programs facilitate Newcastle disease epidemiological studies

*Patti J. Miller[1], Kiril M. Dimitrov[1,2], Dawn Williams-Coplin[1], Melanie P. Peterson[3], Mary J. Pantin-Jackwood[1], David E. Swayne[1], David L. Suarez[1] and Claudio L. Afonso[1]**

[1] Exotic and Emerging Avian Viral Diseases, Southeast Poultry Research Center, United States Department of Agriculture – Agricultural Research Service, Athens, GA, USA, [2] National Diagnostic and Research Veterinary Medicine Institute, Sofia, Bulgaria, [3] Office of International Research Programs, George Washington Carver Center, United States Department of Agriculture – Agricultural Research Service, Beltsville, MD, USA

OPEN ACCESS

Edited by:
Jeanne Marie Fair,
Defense Threat Reduction Agency,
USA

Reviewed by:
Paras Jain,
Albert Einstein College of Medicine,
USA
Li Xu,
Cornell University, USA

***Correspondence:**
Claudio L. Afonso
claudio.afonso@ars.usda.gov

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: 18 June 2015
Accepted: 30 September 2015
Published: 19 October 2015

Citation:
Miller PJ, Dimitrov KM, Williams-Coplin D, Peterson MP, Pantin-Jackwood MJ, Swayne DE, Suarez DL and Afonso CL (2015) International biological engagement programs facilitate Newcastle disease epidemiological studies. Front. Public Health 3:235. doi: 10.3389/fpubh.2015.00235

Infections of poultry species with virulent strains of Newcastle disease virus (NDV) cause Newcastle disease (ND), one of the most economically significant and devastating diseases for poultry producers worldwide. Biological engagement programs between the Southeast Poultry Research Laboratory (SEPRL) of the United States Department of Agriculture and laboratories from Russia, Pakistan, Ukraine, Kazakhstan, and Indonesia collectively have produced a better understanding of the genetic diversity and evolution of the viruses responsible for ND, which is crucial for the control of the disease. The data from Kazakhstan, Russia, and Ukraine identified possible migratory routes for birds that may carry both virulent NDV (vNDV) and NDV of low virulence into Europe. In addition, related NDV strains were isolated from wild birds in Ukraine and Nigeria, and from birds in continental USA, Alaska, Russia, and Japan, identifying wild birds as a possible mechanism of intercontinental spread of NDV of low virulence. More recently, the detection of new sub-genotypes of vNDV suggests that a new, fifth, panzootic of ND has already originated in Southeast Asia, extended to the Middle East, and is now entering into Eastern Europe. Despite expected challenges when multiple independent laboratories interact, many scientists from the collaborating countries have successfully been trained by SEPRL on molecular diagnostics, best laboratory practices, and critical biosecurity protocols, providing our partners the capacity to further train other employes and to identify locally the viruses that cause this OIE listed disease. These and other collaborations with partners in Mexico, Bulgaria, Israel, and Tanzania have allowed SEPRL scientists to engage in field studies, to elucidate more aspects of ND epidemiology in endemic countries, and to understand the challenges that the scientists and field veterinarians in these countries face on a daily basis. Finally, new viral characterization tools have been developed and are now available to the scientific community.

Keywords: newcastle disease, NDV, APMV-1, surveillance, biological engagement programs, wild birds, poultry

INTRODUCTION

Newcastle disease (ND) is one of the most significant diseases of poultry worldwide. It is caused by virulent strains of Newcastle disease virus (NDV), also known as avian paramyxoviruses of serotype 1 (APMV-1). The presence of virulent viruses in poultry must be immediately reported to the World Organisation for Animal Health (1). From 2012 through 2014, infection with virulent NDV (vNDV) was suspected or reported to the OIE by 58 countries as being present in domestic poultry, with an additional 5 countries reporting the disease in limited zones.[1] During this same time period, 15 countries reported vNDV in wild birds to the OIE. Many countries do not adhere to OIE reporting guidelines for vNDV, as the disease is endemic; therefore, the presence of the virus is underreported. The true prevalence of vNDV in the wild-bird population is not reflected in the limited number of reports, since wild-bird species are not routinely studied. Except for studies on migratory birds (which are principally surveyed for the presence of avian influenza) there are few available funds for the surveillance of diseases in other wild-bird species, and logistically sampling wild birds is difficult due to the regulations protecting their capture.

The greatest impact of ND is due to the high mortality rates on village poultry, reducing egg, and meat protein availability. Village or smallholder poultry production is a major source of protein and income for small farmers, and outbreaks of ND contribute to food insecurity in these communities. In addition, ND also affects intensive production facilities worldwide by triggering the implementation of economically significant trade restrictions, and by increasing costs of production from culling and quarantines for the infected premises. vNDV is also considered as a select agent and a potential bioterrorism threat agent in the United States (US). Whether in domestic poultry or wild birds, vNDV strains remain a threat to all producers of poultry.

BIOLOGICAL ENGAGEMENT PROGRAMS

In 1998, the US Department of State (DOS) assisted selected former Soviet chemical and biological weapons scientists to redirect their efforts to peaceful, agricultural research and to help reduce the proliferation of weapons of mass destruction. Almost a decade later, the DOS Biosecurity Engagement Program (BEP) was implemented with the broader mission of providing financial and intellectual assistance to microbiological laboratories to enhance biosecurity, biosafety, and pathogen and disease surveillance, while decreasing biological threats, globally. The collaborations expanded past the former Soviet Union (FSU) to include South and Southeast Asia, the Middle East and Africa. One area of common mutual interest across these regions was scientific collaboration on avian influenza virus (AIV) and NDV, both notifiable and listed diseases to the OIE.

In 2000, a long-standing collaboration between the ARS Southeast Poultry Research Laboratory (SEPRL) and Russian counterparts began on avian influenza and NDV and provided unique opportunities for surveillance and research in the region. In 2002, SEPRL began collaborating with partners in Kazakhstan; in 2010, collaborations began with Ukraine, Egypt and Indonesia; and in 2011, with Pakistan. From 2000 to 2015, SEPRL has collaborated on 14 bio-engagement research projects on avian influenza and ND with its global partners mainly funded by the DOS, but also more recently by the Department of Defense – Defense Threat Reduction Agency. While most of the initial interactions with foreign laboratories often began with the training of collaborators at SEPRL, formal scientific collaborations often developed subsequently (**Table 1**).

The primary role of SEPRL in these collaborations was to analyze, coordinate, integrate, centralize, and share epidemiological information among partners. Another key role was to develop and provide standard protocols and operating procedures to allow data from multiple institutions to be compared. The collaborating institutions provided expertise for each of their localities concerning management practices necessary for field surveillance and sample collections.

Training

The training objectives and goals for foreign collaborating scientists have been diverse and adapted to their respective needs, depending on their country of origin. Representatives of Afghanistan, Azerbaijan, Brazil, Egypt, Georgia, Indonesia, Kazakhstan, Kenya, Libya, Malaysia, Mongolia, Mexico, Morocco, Nigeria, Pakistan, Palestine, Russia, and Yemen have received different levels of training at SEPRL. The BEP collaborations began by building on the success that SEPRL previously established through international collaborations with the International Science and Technology Center (ISTC), Food and Agricultural Organization (3), US Agency for International Development (USAID), Foreign Agricultural Service (4), Animal and Plant Health Inspection Service (5), and Wildlife Conservation Society funded projects collaborating with Japan, South Korea, Indonesia, Vietnam, Russia and FSU countries, e.g., Kazakhstan. These collaborations have built a network of avian influenza and ND scientists around the world, which have yielded joint research projects, published scientific studies in peer-reviewed journals, resolved trade issues on poultry products, and improved competence of all of the scientists involved. Objectives included in all collaborative training modules have improved diagnostic assays and enhancement of biosecurity. For diagnostics, visiting scientists have learned techniques of egg inoculation for virus isolation and identification, real-time reverse-transcriptase PCR (rRT-PCR) for the detection of NDV, and methods of differentiation of vNDV from NDV of low virulence utilizing rRT-PCR. Techniques and basic principles of primer designs, sequencing, sample preparation, phylogenetic tree construction, and serology (hemagglutination-inhibition [HI] assay and ELISA) were transferred during the collaboration. Biosecurity and containment operations training included laboratory safety, proper safety data records, handling compressed gas cylinders, Occupational Safety Health Administration (OSHA) laboratory standards, general biosecurity, biohazard risk assessment and management, disinfection and sterilization, personal protective equipment, emergency and spill response, engineering

[1] http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist.

TABLE 1 | Contact information and country of origin for all collaborating institutions.

Institution contact information	Country
National Diagnostic and Research Veterinary Medicine Institute, 15 Slaveikov Blvd, Sofia 1606, Bulgaria	Bulgaria[a]
Laboratory of the Ministry of Agriculture, Tbilisi Laboratory of the Ministry of Agriculture (2), 65 Godziashvili str, Tbilisi 0159, Georgia	Georgia
Faculty of Veterinary Medicine, Bogor Agricultural University, Jl. Agatis Kampus IPB Dramaga, Bogor 16880, West Java, Indonesia	Indonesia
Gadjah Mada University, Faculty of Veterinary Medicine, Jl. Fauna 2, Karang Malang, Yogyakarta 55281, Indonesia	Indonesia
Kimron Veterinary Institute, Israel, Division of Avian Diseases, Bet Dagan, P. O. Box 12, Israel 50250	Israel[a]
Institute of Microbiology and Virology, 103, Bogenbai batyr Str., 480100, Almaty, Kazakhstan	Kazakhstan
Research Institute of the Biosafety Problems (RIBSP) 19–13, Gvardeisky, Kordaiskiy 080409, Kazakhstan	Kazakhstan
Comisión México Estados Unidos para la prevención de Fiebre Aftosa, Senasica Rio Pánuco #852 Fracc, Los Laguitos, Edificio SAGARPA C. P. 29029 Tuxtla Gutiérrez, Chiapas Mexico	Mexico
National Veterinary Research Institute, PMB 01 Vom, Plateau State, Nigeria	Nigeria[a]
Hivet Animal Health Business 667-P, Johar Town, Lahore, Pakistan, 45000	Pakistan
Quality Operations Laboratory/Institute of Biochemistry & Biotechnology, University Of Veterinary and Animal Sciences, Lahore, Pakistan 45000	Pakistan
D.I. Ivanovsky Virology Institute, Minzdravsocrazvitia Rossii, Scientific Production Association "Narvac" 16 Gamaleyi St., Moscow, Russia, 123098	Russia
"Federal Centre for Animal Health" (FGI "ARRIAH"). 600901 Yur'evets, Vladimir, Russia	Russia
Novosibirsk State University, Department of Research, Novosibirsk, Russia 630090, (383)–330–3244, and Division of Emerging Zoonotic Diseases and Influenza. State Research Center of Virology and Biotechnology "Vector," 630559, Koltsovo, Novosibirsk region, Russia	Russia
Department of Microbiology and Biotechnology, School of Biological Sciences, University of Dodoma, P. O. Box 259, Dodoma, Tanzania	Tanzania
Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, P. O. Box 3021, Chuo Kikuu, Morogoro, Tanzania	Tanzania
National Scientific Centre, "Institute of Experimental and Clinical Veterinary Medicine," 83, Pushkinskaya Street, Kharkiv 61023, Ukraine	Ukraine
Emerging and Exotic Avian Viral Disease Research Unit, Southeast Poultry Research Laboratory, US National Poultry Research Center, USDA/ARS, 934 College Station Road, Athens, GA 30605, USA	USA

[a]*Collaboration not funded by BEP.*

controls, and shipping regulated biological materials. For a selected group of scientists that received extended training, other modules offered included working with the Institutional Animal Care and Use Committee (IACUC), which allowed them to be included on animal use protocols and to be involved with animal studies.

During the research projects, SEPRL scientists visited their foreign counterparts in Russia, Kazakhstan, and Ukraine 32 times. SEPRL scientists also hosted 30 collaborators for scientific visits involving research and training. Under the auspices of the projects, SEPRL and foreign collaborators participated in over 25 international conferences in the US, Russia, Ukraine, South Africa, Spain, Germany, China, Latvia, the United Kingdom, and Thailand. In addition to the research projects, from 2008 to 2010, SEPRL provided training on molecular techniques to detect AIV and NDV to 57 scientists from 21 countries. Additional informal collaborations with scientists from Israel, Bulgaria, Nigeria, Tanzania, and Mexico have added to the body of knowledge on AIV and NDV epidemiology. Through training and collaborative research efforts, SEPRL has developed an extensive network of scientists around the world working on AIV and NDV.

The initial objective of the scientific proposals between SEPRL and the collaborating laboratories included increasing the number of surveillance samples obtained from commercial and domestic poultry species, and also wild birds. Scientists from the participant laboratories processed these samples together with SEPRL scientists, identified and characterized the strains of NDV obtained. Subsequent testing of molecular diagnostic techniques documented if routinely used molecular assays were able to detect all of the viruses isolated. Additional characterization of isolates, such as pathogenesis studies in chickens, vaccine studies, or phylogenetic sequence analysis was performed depending on the scientific interests or industry concerns. The objective of the training was to prepare foreign scientists to be able to accurately and promptly detect vNDV and to identify what avian species the viruses are likely to be obtained from. Those skills were considered to be critical for preventing, containing, and controlling ND outbreaks. Furthermore, efforts were made in promoting safe, secure, and responsible use of biological materials that are at risk of accidental release or intentional misuse as the prevention of accidents improves the quality of life for people from all the countries involved.

Molecular Epidemiology of Lentogenic Viruses

NDV of low virulence, also known as lentogenic NDV, are widely distributed worldwide and are present in multiple avian species that may be in contact with poultry. Lentogenic viruses are used as vaccines to prevent mortality and clinical disease from vNDV infections in poultry. However, some lentogenic strains produce respiratory infections that decrease productivity in poultry, and have the potential to become virulent upon mutation of the cleavage site of the fusion protein gene. The nucleotide sequence of the fusion gene is used to classify NDV strains into genotypes and the cleavage site sequence of the fusion protein of NDV is the accepted OIE identifier of virulence. Strains that have three or more basic amino acids at positions 113–116 with a phenylalanine at position 117 are by definition virulent (1). Since scientists began to analyze NDV strains phylogenetically, the fusion gene nucleotide sequence has been and continues to be the standard gene used to classify NDV strains and to study virus evolution (6, 7). Although infection with vNDV is one of the greatest concerns for poultry producers, a better understanding of the epidemiology of lentogenic NDV is also very important, as transmission of these viruses from poultry to wild birds, and vice versa, has been previously documented. The distribution of lentogenic NDV in wild birds has been studied by our group and by others, however, the full extent of the interactions between lentogenic NDV from

wild birds and poultry is largely unknown (8–10). As most lentogenic viruses are normally detected using the same diagnostic assays (serology and real-time PCR) as virulent viruses, the interpretation of some previously published epidemiological data, that does not involve sequencing or that distinguish vNDV from NDV, is difficult. Thus, efforts have been made to train and to develop sequencing capacities in partner countries.

A collaboration project with the National Scientific Center Institute of Experimental and Clinical Veterinary Medicine, Kharkiv, Ukraine was initiated to study the geographic distribution, wild host species distribution, and ecological factors affecting virus transmission of avian paramyxoviruses in Ukraine. Wild-bird surveillance was conducted in Crimea, Kherson, Zaporizhia and the Donetsk regions of Ukraine. The Black and Azov Sea regions are part of an intercontinental (north to south and east to west) flyway, and the similarity of the NDV strains isolated from these regions with other NDV from Northern Europe and Africa confirmed the importance of sampling birds in these regions for the early detection of viruses transported by wild birds (11).

Surveillance for hemagglutinating (HA) avian paramyxoviruses was conducted during 2006–2011 through different seasons of the year on 6,735 wild birds, representing 86 species, from 8 different orders. The presence of HA positive viruses in oral and cloacal swabs was obtained and the serotypes of the APMV were identified by hemagglutination-inhibition tests. The APMV obtained from swabs were serologically characterized and determined to belong to different serotypes as follows: APMV-1 ($n = 9$), APMV-4 ($n = 4$), APMV-6 ($n = 3$), and APMV-7 ($n = 4$) (11). Overall the highest viral isolation rate occurred during north to south autumn migration with viruses isolated mainly from mallards, teals, dunlins, and a widgeons at rates ranging from 1.9 to 25% depending on species and location. The rate of isolation was lower during winter (December–March) (0.32%), with viruses isolated mainly in the Black and Azov Sea regions from ruddy shelducks, mallards, white-fronted geese, and a starling (12). Surprisingly, during spring migration, and the time that included nesting (April–August), no APMV strains were isolated out of 1,984 samples tested. Sequencing and phylogenetic analysis of four APMV-1 and two APMV-4 viruses showed that one APMV-1 virus belonging to class I was epidemiologically linked to viruses from China, three class II APMV-1 viruses were epidemiologically connected with viruses from Nigeria and Luxembourg, and one APMV-4 virus was related to goose viruses from Egypt. In summary, multiple wild-bird species likely to be infected with different types of APMVs were identified, and the data supported the existence of a possible Africa-Europe route of intercontinental transmission of APMVs by wild birds.

As a result of an international collaboration that included the US Geological Survey at the Alaska Science Center in Anchorage, the Southeastern Cooperative Wildlife Disease Study group of The University of Georgia, Athens, GA, USA, the Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine in Inada, Hokkaido, Japan, and the State Research Center of Virology and Biotechnology 'VECTOR', Novosibirsk Region, Koltsovo, Russia, the genetic diversity of APMV-1 isolated from migratory birds connected with Alaska and the continental US was studied (13). Swabs samples from migratory birds from the US, Japan and Russia were assessed for the evidence for north-south and east-west intercontinental virus spread. Viruses were isolated and sequenced, phylogenetic methods of tree construction based on maximum likelihood and prediction of viral virulence by sequence analysis were utilized to compare isolates of APMV-1 strains isolated from migratory birds from Alaska, Japan, and Russia. A total of 73 APMV-1 isolates were sequenced as part of this study. Most isolates were closely related to lentogenic viruses from class I and II genotypes previously reported to be present in wild birds (8).

Analysis of the fusion protein sequence data revealed that none of the putative amino acid sequences for the fusion protein cleavage sites were consistent with those of previously identified virulent viruses. Besides the fact that five isolates of genotype I of class II formed a monophyletic cluster exhibiting previously unreported genetic diversity, which met criteria for the designation of a new sub-genotype, the most significant finding of this study was the close genetic relationship among selected isolates from wild-bird isolates from widely divergent geographic location. Close relationships were found among viruses of class II, with Russian NDV strains closely related to North American viruses (class II sub-genotypes Ib and Ic). More specifically NDV strains from Alaska (US) and Japan were related to NDV from Maryland (US), suggesting multiple opportunities where birds from different areas were exposed to each other's viruses. The relatedness of these viruses provided indirect evidence for possible intercontinental virus spread by migratory birds. Furthermore, the close relationship between NDV from class II genotype I, sub-genotypes Ib and Ic, confirmed that migratory bird movement is one of the possible mechanism for the redistribution of NDV strains (13). Due to the location and the intensity of the surveillance, most class I viruses were from North America. Interestingly, two isolates, one each from Japan and Russia were closely related to American viruses of class I, sub-genotype 1c. These data, however, did not provide support for the hypothesis that wild-bird strains may be contributing to the emergence of more vNDV as, none of the isolates resembled known virulent viruses. The estimated mutation rates for fusion genes of class I and class II wild-bird strains were within the values typical for NDV strains and there was no evidence of vaccine viruses present in waterfowl. Thus, these studies provided new insights into the diversity, spread, and evolution of lentogenic NDV in migratory wild birds, worldwide.

Epidemiology of vNDV Strains that Cause ND

The epidemiological factors responsible for the maintenance and spread of vNDV strains are largely unknown. It is suspected that vNDV may persist in vaccinated animals, however, repeated isolations of vNDV strains in wild birds also suggests the existence of natural reservoirs in birds species that either might not be susceptible to disease or may be partially resistant, such as parrots, cormorants and pigeons. Evaluating surveillance samples of domestic poultry species along with samples from wild-bird

populations geographically surrounding them is critical for completely understanding the epidemiology of ND.

The BEP collaborations between SEPRL and Pakistan involved two departments of the University of Veterinary and Animal Sciences, in Lahore; (1) Quality Operations Laboratory and (2) the Pakistan Institute of Biochemistry and Biotechnology, and another institution, the Poultry Disease Diagnostic Laboratory in Gakkhar, Gujranwala, Punjab. Efforts were focused on epidemiological studies designed to identify and characterize circulating strains of NDV in Pakistan. The first BEP collaboration with

TABLE 2 | Primer pairs used for sequencing the complete fusion gene of different Newcastle disease viruses from class I and class II.

Primer pairs used for sequencing	CLASS II sub/genotype[1]																CLASS I
	I[a]	I[b]	I[c]	II	III	V[b]	VII	VII[b]	VII[d]	VII[i]	XII	XIII[b]	XIV[b]	XVI	XVII	XVII[b]	
MSF1/NDVR2	X		X	X	X	X	X				X	X					X
4331F/5090R[2]	X	X	X	X		X	X				X	X	X	X	X	X	X
4093F/4889R	X																
4707F/5518R	X		X														
4961F/5772R[2]	X	X	X	X		X							X	X	X		
4927F/5673R						X	X				X	X					
5358F/6178Ra	X		X														
5435F/6320R		X	X	X	X	X						X	X		X		
5491F/6341R					X		X				X	X					
5413F/6179R														X			
4317F/5078R				X													
4911F/5857R[3]				X													
5669F/6433R				X													
4008F/4994R								X	X	X							
4715F/5637R								X	X	X							
5410F/6332R						X		X	X	X		X					
4715F/6178Rb									X								
4963F/5832R						X											
5633F/6474R						X											
4927F/5641R					X	X	X					X				X	
5378F/6204R						X	X									X	
4504F/4859R										X							
4853F/5324R										X							
5258F/5609R										X							
5592F/5939R										X							
4353F/5129R										X		X					
5067F/5837R												X					
4089F/4895R										X							
4772F/5533R										X							
5412F/6311R										X							
4445F/5098R																	X
5969F/5748R																	X
5550F/6326R																	X
5550F[4]/6326R[5]																	X
5076F/5838R																	X
5643F/6354R																	X

[1]*The table represents primer pairs used in this manuscript to identify each genotype. Primer pairs not marked with an X may also identify isolates from other genotypes.*

[2]*Primers designation as published previously (8, 28).*

[3]*The use of this set of primers did not result in PCR products for all tested genotype II isolates.*

[4,5]*Modified by Andrew Reeves (13).*

The sequences of all primers are presented in ***Table 3***.

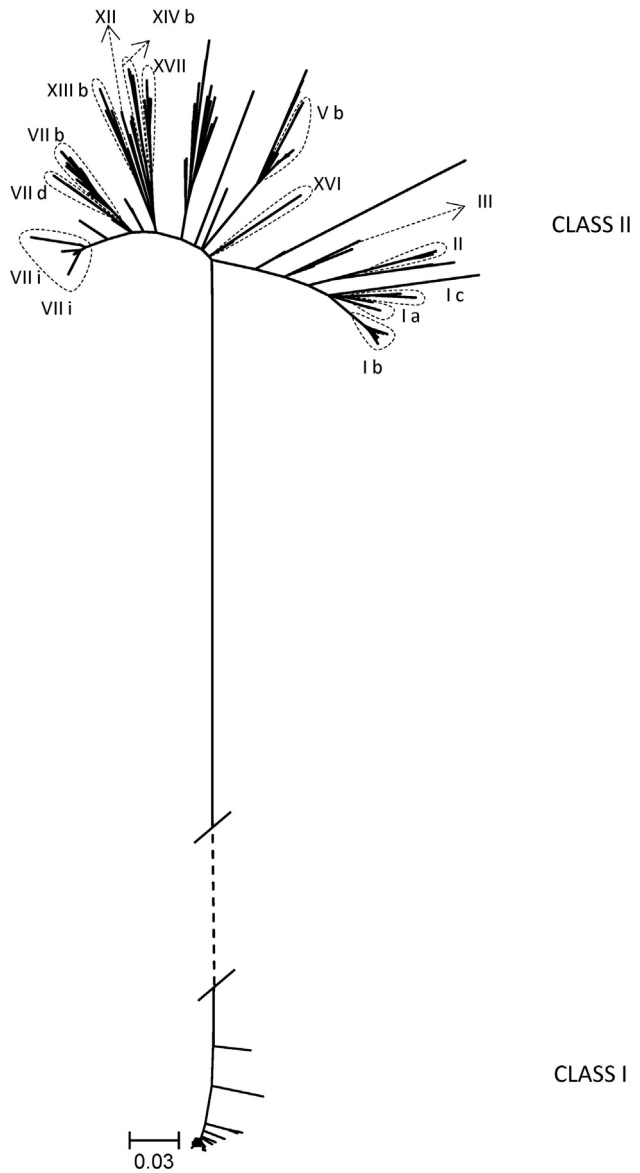

FIGURE 1 | Phylogenetic tree of complete coding sequences of the fusion gene of all viruses studied within the Biological Engagement Programs and additional collaborations ($n = 183$). Selected viruses from all known genotypes in class I and class II are also included in the tree ($n = 94$). The evolutionary history was inferred by using the Maximum Likelihood method based on General Time Reversible model as implemented in MEGA6 with 500 bootstrap replicates (29). The branch lengths represent the number of substitutions and are proportional to the differences between the isolates. The branch between class I and class II (dashed) is not drawn to scale. Evolutionary analyses were conducted in MEGA6 (30).

Pakistan characterized eight vNDV strains isolated from the years 1974, 1995, and 2004 through 2008 (14). Besides characterizing by sequencing a 374 base pair (15) region of the fusion gene that is critical to determine virulence, the mean death time (MDT) and intracerebral pathogenicity index assay (ICPI) were performed to confirm their virulence. The isolates from 2007 and 2008 grouped into distinct cluster within genotype VII and were most closely related to a vNDV isolated from Japan in 1989. These viruses later proved to be an entirely distinct genotype XIII (7), later noted as XIIIb (16). The original NDV strains of genotype XIII (now renamed sub-genotype XIIIa) from South Africa, Iran and India may have given rise to an additional sub-genotype (XIIIb) now present in Pakistan. Intracerebral pathogenicity and assessment of gross and histological assessment of representative strains for sub-genotype VIIi and genotype XIIIb show intracerebral pathogenicity indices (ICPI) of 1.89 and 1.85, and 100% mortality within 4 and 5 days, respectively (17). The ICPI scale goes from 0 to 2, with a score close to zero meaning no morbidity or mortality, a score equal to or greater than 0.7 meaning a virulent and reportable virus, and a score closer to 2 meaning a highly virulent virus in poultry (1). Therefore, the Pakistani viruses were highly virulent.

The majority of the remaining Pakistani strains grouped with variants of NDV found in pigeons from genotype VIb, often called pigeon paramyxovirus-1 (PPMV-1). PPMV-1 are virulent by definition since they contain three or more basic amino acids at positions 113–116 and a phenylalanine at position 117 of the fusion protein cleavage site and have an ICPI of equal to or greater than 0.7, thus are reportable to OIE (1). While these isolates are found worldwide in pigeons, they show varying rates of clinical disease upon infecting chickens. The Pakistani collaborations have continued and remain on going. As a result, besides genotypes XIIIb and VIb two other NDV genotypes were identified from circulating vNDV at different times in Pakistan and the surrounding countries. These included vNDV strains of the most widely distributed genotype VIId, and a newer sub-genotype VIIi that have been found in Pakistan and the Middle East.

Most recently, our international collaborations have broadened to include collecting data to improve the current knowledge concerning the mobility and evolution of NDV genotypes circulating since 2011. These studies incorporated collaborations with the Kimron Veterinary Institute, from Bet Dagan Israel, and the Department of Infectious Diseases and Veterinary Public Health, of the Veterinary Medicine-Bogor Agricultural University, in Bogor, Indonesia. These studies lead to the discovery that vNDV had rapidly spread across continents into three countries that are not geographically connected (16). Viruses of sub-genotype VIIi were found to be rapidly spreading through Asia and the Middle East causing outbreaks of ND in vaccinated birds, wild birds, and pets (18). A close phylogenetic relationship with viruses previously isolated in wild birds in Indonesia was found and it was suggested that permanent uncharacterized reservoirs of NDV might exist in wild birds. The discovery of viruses of sub-genotype VIIi in vaccinated animals (19) suggested that this new sub-genotype has the potential to be maintained in vaccinated animals. Additionally, the presence of significant disease on farms since 2011 until 2013 throughout Indonesia, Pakistan, and Israel caused by viruses of this sub-genotype highlight its potential to cause a new panzootic (16). Further evidence supporting these findings is the introduction of this virus into Eastern Europe (16).

Another new sub-genotype, VIIh, has also been identified in Indonesia, Bali, China, and Malaysia from viruses isolated from 2007 through 2011. Most significant is the fact that the viruses of new genotypes seem to be replacing older viruses. In Pakistan,

TABLE 3 | Sequences of the primer pairs used for sequencing the complete fusion gene of different Newcastle disease viruses from class I and class II.

Primer pair	Sequence (5′–3′)	Tm in °C	Reference
MSF1/NDVR2	GACCGCTGACCACGAGGTTA AGTCGGAGGATGTTGGCAGC	55.9 55.9	(6)
4331F/5090R[1]	GAGGTTACCTCYACYAAGCTRGAGA TCATTAACAAAYTGCTGCATCTTCCCWAC	56–61 57.3– 58.7	(8)
4093F/4889R	ATCTGTCGGGCTCAGCGACGTG AGCGCCTATAAGGCGTCCCTG	60.4 58.3	
4707F/5518R	ACACCTCATCCCAGACAGGGTCG CTATCACGGAACCGACCTGCGTC	60.6 60.6	
4961F/5772R[1]	GCTCTGATACARGCMAAMCAAA TGCGATATGATWCCCGGRG	49.2–54.8 51.1–53.2	(28)
4927F/5673R	TCTTGGGGTTGCAACAGCGGCAC GGCGTAGTGAGTGCACCTTCAG	60.6 58.6	
5358F/6178Ra	CCGGCAACCCTATCCTGTACGAC AGTGGCCCTCATCTGGTCCAGG	60.6 60.4	
5435F/6320R	AAYAATATGCGYGCCACCTACYTRG ACCGTTCTACCCGTRTRTYGY	54.4–61 50.5–58.3	
5491F/6341R	TGCCTCAGCACTTGTCCCGAAAG TCGATTGAAGGATGGCTCCTCTG	58.8 57.1	
5413F/6179R	TCTACCCTCAGTTGGGAACC GTTGGCCCTCATCTGATCGAG	53.8 56.3	
4317F/5078R	CCGACCACGAGGTTACC TGCATCTTCCCAACTGCCAC	51.9 53.8	
4911F/5857R	TTATTGGCGGTGTGGCTC AGTTACATCGAATTCCCCACTG	50.3 53	
5669F/6433R	AAGACCGAAGGCGCACTTAC TCTCTAACGCAACTTGGCT	53.8 48.9	
4008F/4994R	ATATCGGGCTTATGTCCACTG CTTAAGCCGGAGGATGTTGGC	52.4 56.3	
4715F/5637R	TCTCAGACAGGGTCAATC AAGCTGACGTATTGCCGCTCA	48 54.4	
5410F/6332R	GAATTTGCCCTCAGTCGGGA GTGGCTCCTCTGACCGTTCTA	53.8 56.3	(16)
4715F/6178Rb	TCTCAGACAGGGTCAATC GTAGTGGCTCTCATCTGATCGAGG	48 59.1	
4963F/5832R	TGCAGCTCTGATACAAGCCAACC AGCCTCAGGGTTATTCCGTCTAGGG	57.1 61	
5633F/6474R	TGTCTGAGCGGTAATACGTCAGCTTG TGATCCGAAAAACCAAGCGCCATGTG	59.5 59.5	
4927F/5641R	TCTTGGGGTTGCAACAGCGGCAC CATGCACGCTGACGTATTGCCG	60.6 58.6	
5378F/6204R	GACTCACAGACTCAACTCTTGG CTCTCATCTGCGTTCATGCTC	54.8 54.4	
4504F/4859R	ACGGGTAGAAGATTCTGGATC CCTCCTGATGTGGACACAGCCCC	52.4 62.4	(16)
4853F/5324R	ATACAGGGGGCTGTGTCCAC CTACCAATTAATGAGCTGAGTTG	55.9 51.7	(16)
5258F/5609R	GCACTTTACAATCTAGCTGGT ATACCAGGGGACATAGG	50.5 47.1	(16)
5592F/5939R	CAAGAATAGTAACATTCCCTATG ACATTCCCAAGCTCAGTTGA	49.9 49.7	(16)
4353F/5129R	GGCACACCATTGCTAAATAC TATACAATCCAATTCTCGCGC	49.7 50.5	(16)
5067F/5837R	CACAACTAGCAGTGGCAGT AGCCTCAGAGTTATCCCGTC	51.1 53.8	(16)
4089F/4895R	ATCTATCTGTCGGGCTCAGTGAC GCCATTAACGGCACCTATAAAGCG	57.1 57.4	(16)

(Continued)

TABLE 3 | Continued

Primer pair		Sequence (5′–3′)	Tm in °C	Reference
4772F/5533R		GCGTGTGCAAAAGCCCCATTAG GAGGTGTCAAGTTCTTCTATCACG	56.7 55.7	(16)
5412F/6311R		ATTTGCCCTCAGTCGGGAACC ACCCGTGTATCGTTCTTTGGTC	56.3 54.8	(16)
4445F/5098R[2]		CACTAATCAAGTCTGATAATTGAACC GTCRTTAACAAACTGCTGCATC	53.2 51.1–53	
5969F/5748R[1,2,3]		TTCTGCCCTCATACAAGCCAACC GGGGGATCTGCGCACCTACACG	57.1 62.3	
5550F/6326R[2]		AGCTGGATACGTCATATTGCATAG ACCGTTCTACCCGTRTATCGY	54 52.4–56.3	
5550F[2,3]	1 abr 2 abr	AGCTGGATACATCGTATTGCATAG AACTGGATACGTCATATTGCATAG	54 52.3	Andrew Reeves, personal communication
6326R[2,3]	1 abr 2 abr 3 abr	ACTGTTCTACCCGTATATCTT ACTGTTCTACCCGTATATATC ACYGTTCTACCCGTRTRTYGY	48.5 48.5 48.5–58.3	Andrew Reeves, personal communication
5076F/5838R[2]		AGCTGGCTGTTGCCGTAGGT CGCAAGGTGATCCCGTCGAGAG	55.9 60.4	
5643F/6354R[2]		TCGGTGGAAACACCTCAGCATGC ACGCTCCTTGAATGGAGGCGAC	58.8 58.6	

[1]*Primers designation as published previously (8, 28).*

[2]*Aligned to the complete genome of APMV-1/Goose/Alaska/415/91 (GenBank Acc.# AB524405) and designated accordingly.*

[2,3]*Modified by Andrew Reeves (13).*

T_m*, melting temperature.*

All primers (except class I[b] primers) were aligned to the complete genome of APMV-1/Goose/China/ZJ1/2000 (GenBank Acc.# AF431744.3) and designated accordingly.

the viruses of sub-genotype VIIi have replaced NDV isolates of genotype XIII, which were commonly isolated in 2009–2011, and they have become the predominant sub-genotype causing ND outbreaks since 2012. Similarly viruses of sub-genotype VIIi were isolated in Israel in 2012, while the presence of the previously predominant sub-genotypes VIId and VIIb, decreased during that year.

Although the viruses of one of the most commonly isolated sub-genotypes of genotype VII (VIId) has been gradually replaced by new viruses in some countries of Asia and the Middle East, these older viruses of VIId have continued to circulate worldwide. Three viruses isolated from chickens in Ukraine between 2003 and 2013 have been classified as members of sub-genotype VIId. Interestingly, these viruses resembled NDV isolates from back-yard poultry in Bulgaria from 2006 and 2007 (unpublished data). These viruses were closely related to isolates from China from 2003 to 2007 and Serbia from 2007, further providing evidence for the distribution patterns of vNDV.

The earliest reports of ND outbreaks in Kazakhstan were reported in 1980 and despite regular vaccination of domestic poultry the disease is thought to be endemic in the country (2). Twenty-eight vNDV strains from chickens from Kazakhstan and Kyrgyzstan isolated from 1998 through 2005 were found to have virulent fusion protein cleavage sites (^{113}-R-Q-R/K-R-F-^{117}) and ICPI values ranging from 1.05 to 1.87 (20). Similar to what happened in Pakistan; the strains isolated from chickens in 1998 through 2001 of sub-genotype VIIb were replaced by strains from sub-genotype VIId from 2003 onward. Altogether, during this collaboration 38 chicken strains were sequenced and characterized. Additional work on three variant NDV strains (PPMV-1) from a 2005 ND outbreak in pigeons found these strains to be similar to other classical PPMV-1 of sub-genotype VIb clustering with other mesogenic (moderately virulent) viruses from Poland, Austria, and Croatia (21).

Similarly, our Russian collaborators evaluated 77 strains from sick or dead domestic and feral pigeons collected from 2001 through 2009 from 17 administrative divisions. While seven of these strains grouped with the same sub-genotype, VIb as the strains from Kazakhstan, 70 of the strains grouped into a different sub-genotype of VIb with 52 located in the European portion of the country and 18 in the more easterly Siberian portion (22). With ICPI values ranging from 0.80 to 1.4, these strains were also considered to be mesogenic. The presence of one of the Siberian strains from a bird in the European portion of the country demonstrates that viruses of pigeons do move across large distances regardless of geographical borders.

USDA offshore funding to study the epidemiology of vNDV in Mexico allowed the identification of vNDV from a sub-genotype Vb, circulating in poultry. Furthermore, the isolation of NDV of low virulence, commonly used as live NDV vaccines in several species of wild birds, provided evidence to suggest the existence of epidemiological links and more than one spillover from poultry into the environment (10). Additional characterization of circulating viruses in Mexico and Central America allowed designation of a new sub-genotype, namely Vc. Three Belize isolates from 2008 were nearly identical to a Honduras isolate from 2007 (sub-genotype Vb), but distinct from viruses circulating in Mexico (also sub-genotype Vb) during the same time

period, thus suggesting separate evolution among viruses from Mexico and Central America (23).

An additional collaboration with Tanzania analyzed surveillance samples from live bird markets in five different areas of the country with vNDV isolated from 10% of the samples and further characterization of these samples is ongoing (unpublished data).

Thus, the evidence suggests that the simultaneous evolution of vNDV strains from multiple genotypes has continued in different locations and different hosts since the first vNDV were identified in 1926. However, increased surveillance, and prompt epidemiological characterization of new strains circulating in developing countries funded by BEP and other international efforts have allowed a better understanding of the evolution of vNDV and has increased awareness that ND is a global problem.

Diagnostics

One of the goals of the BEP collaborations was the evaluation of current diagnostic methods against new emerging strains of NDV. The constantly developing network of laboratories in different countries around the world, collaborating on the control of the ND, resulted in the increasing number of samples obtained from both poultry (commercial and backyard) and wild birds. Thousands of collected specimens where tested by various serological, virological, and molecular methods. As a result, more than 350 NDV strains were isolated and most of them have been further studied and characterized. Most currently utilized rapid diagnostic methods based on rRT-PCR were effective in detecting ND viruses. The L-TET rRT-PCR designed to detect class I APMV-1 viruses (8) and multiplex rRT-PCR designed to detect both class I and class II APMV-1 viruses (9) have been successfully used for ND diagnostics. Furthermore, the modified fusion gene rRT-PCR (24) has been routinely applied in detecting of pigeon PMV-1 (25). However, the matrix gene rRT-PCR assay universally used and designed to detect all NDV class II strains (26), did not detect three isolates from Pakistan from 2006 to 2007 (14). A new matrix gene test with a modified probe that detected these isolates was developed (14). These viruses were later identified as members of a new genotype – XIII (7).

An additional goal of the BEP collaborations was the development of tools and knowledge to better understand the genetic evolution and molecular epidemiology of ND. Initial efforts were directed to obtain nucleotide sequences of different regions of the isolated strains' genomes. In total, 222 partial fusion gene sequences from viruses from Indonesia, Kazakhstan, Kyrgyzstan, Mexico, Nigeria, Pakistan, Russia, and Ukraine and 18 matrix gene sequences from viruses from Pakistan and Mexico were obtained (18, 20, 22) (unpublished data). As the complete fusion gene sequence was later shown to provide more detailed and valuable information on ND epidemiology and NDV classification (7), this section of the NDV genome from 187 viruses was also sequenced. These viruses were collected from poultry and wild birds in Bulgaria, Indonesia, Israel, Japan, Malaysia, Mexico, Nigeria, Pakistan, Russia, U.S., and Ukraine and represented 17 different sub/genotypes within class I ($n = 52$) and class II ($n = 135$) (7, 10, 11, 13, 16, 23, 27) (unpublished data) (**Table 2**; **Figure 1**). The high diversity of viruses studied within the BEP and additional collaborations are represented in a phylogenetic tree including selected isolates from all known genotypes (**Figure 1**). As NDV is continuously evolving and leading to more diversity, new primers have been designed and tested to facilitate the sequencing of the complete fusion gene. The newly designed primers and also some previously published ones are presented in **Tables 2** and **3**, including the utilization scheme by sub/genotype. The availability of those primers should facilitate future detailed genetic characterization of NDV isolates.

CONCLUSION

Newcastle disease represents an international problem and a significant threat to poultry industries worldwide. The existence of a large number of countries with endemic virulent ND circulating in poultry and wild birds, the capacity of viruses for rapid intercontinental spread, the ability of viruses to gradually change, and the potential use for bioterrorism, require the development of more internationally funded epidemiological and veterinary research programs. The BEP collaborative projects have effectively contributed to the understanding of the threat and to the development of better tools to identify and characterize this agent.

AUTHOR CONTRIBUTIONS

PM, KD, and CA defined the theme of the manuscript. All authors contributed on the data collection, writing of the manuscript and editing the manuscript. KD, PM, and DW-C created the tables. KD created the figure. DW-C, KD, and CA designed primers. All authors have seen and approved the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank the technicians who helped obtain the data and the animal caretakers who help with the animal studies; Tim Olivier, Diane Smith, Suzanne DeBlois, Roger Brock, and Keith Crawford. In addition, the authors are grateful for the opportunity to work with our BEP collaborators listed in **Table 1**, without whom this work would not have been possible. This work was funded by the Department of State Biosecurity Engagement Program (BEP) and the Defense Threat Reduction Agency Cooperative Biological Engagement Program and CRIS 6612-32000-064-00D.

REFERENCES

1. OIE. Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. *Biological Standards Commission [Internet]*, Vol. 1, Part 2, Chapter 2.03.14 (2012). pp. 1–19. Available from: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.
2. Bogoyavlenskiy A, Berezin V, Prilipov A, Usachev E, Lyapina O, Levandovskaya S, et al. Molecular characterization of virulent Newcastle disease virus isolates from chickens during the 1998 NDV outbreak in Kazakhstan. *Virus Genes* (2005) **31**(1):13–20. doi:10.1007/s11262-004-2195-2
3. Snoeck CJ, Owoade AA, Couacy-Hymann E, Alkali BR, Okwen MP, Adeyanju AT, et al. High genetic diversity of Newcastle disease virus in poultry in West

and Central Africa: cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. *J Clin Microbiol* (2013) **51**(7):2250–60. doi:10.1128/JCM.00684-13
4. Fasina FO, Bisschop SP, Joannis TM, Lombin LH, Abolnik C. Molecular characterization and epidemiology of the highly pathogenic avian influenza H5N1 in Nigeria. *Epidemiol Infect* (2009) **137**(4):456–63. doi:10.1017/S0950268808000988
5. USDA. *Exotic Newcastle Disease Emergency Disease Guidelines*. Hyattsville, MD: USDA, APHIS (1992).
6. Aldous EW, Mynn JK, Banks J, Alexander DJ. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathol* (2003) **32**(3):239–56. doi:10.1080/030794503100009783
7. Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infect Genet Evol* (2012) **12**(8):1770–9. doi:10.1016/j.meegid.2012.07.012
8. Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. *J Clin Microbiol* (2007) **45**(4):1310–4. doi:10.1128/JCM.02594-06
9. Kim LM, Suarez DL, Afonso CL. Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. *J Vet Diagn Invest* (2008) **20**(4):414–25. doi:10.1177/104063870802000402
10. Cardenas Garcia S, Navarro Lopez R, Morales R, Olvera MA, Marquez MA, Merino R, et al. Molecular epidemiology of newcastle disease in Mexico and the potential spillover of viruses from poultry into wild bird species. *Appl Environ Microbiol* (2013) **79**(16):4985–92. doi:10.1128/AEM.00993-13
11. Muzyka D, Pantin-Jackwood M, Stegniy B, Rula O, Bolotin V, Stegniy A, et al. Wild bird surveillance for avian paramyxoviruses in the Azov-black sea region of Ukraine (2006 to 2011) reveals epidemiological connections with Europe and Africa. *Appl Environ Microbiol* (2014) **80**(17):5427–38. doi:10.1128/AEM.00733-14
12. Muzyka D, Pantin-Jackwood M, Spackman E, Stegniy B, Rula O, Shutchenko P. Avian influenza virus wild bird surveillance in the Azov and Black Sea regions of Ukraine (2010-2011). *Avian Dis* (2012) **56**(4 Suppl):1010–6. doi:10.1637/10157-040912-ResNote.1
13. Ramey AM, Reeves AB, Ogawa H, Ip HS, Imai K, Bui VN, et al. Genetic diversity and mutation of avian paramyxovirus serotype 1 (Newcastle disease virus) in wild birds and evidence for intercontinental spread. *Arch Virol* (2013) **158**(12):2495–503. doi:10.1007/s00705-013-1761-0
14. Khan TA, Rue CA, Rehmani SF, Ahmed A, Wasilenko JL, Miller PJ, et al. Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *J Clin Microbiol* (2010) **48**(5):1892–4. doi:10.1128/JCM.00148-10
15. Hanson BA, Swayne DE, Senne DA, Lobpries DS, Hurst J, Stallknecht DE. Avian influenza viruses and paramyxoviruses in wintering and resident ducks in Texas. *J Wildl Dis* (2005) **41**(3):624–8. doi:10.7589/0090-3558-41.3.624
16. Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, et al. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infect Genet Evol* (2015) **29**:216–29. doi:10.1016/j.meegid.2014.10.032
17. Pandarangga P, Brown CC, Miller PJ, Haddas R, Rehmani SF, Afonso CL, et al. Pathogenesis of new sub-genotypes of Newcastle disease virus strainsfrom Israel and Pakistan. *Vet Pathol* (2015) **29**:216–29. doi:10.1016/j.meegid.2014.10.032
18. Rehmani SF, Wajid A, Bibi T, Nazir B, Mukhtar N, Hussain A, et al. Presence of virulent newcastle disease virus in vaccinated chickens in farms in pakistan. *J Clin Microbiol* (2015) **53**(5):1715–8. doi:10.1128/JCM.02818-14
19. Wajid A, Muhammad W, Rehmani SF, Bibi T, Ahmed N. Complete genome sequence of a new panzootic strain of virulent Newcastle disease virus isolated from a recent outbreak in chicken in Pakistan. *Genome Announc* (2015) **3**(3):e658–615. doi:10.1128/genomeA.00658-15
20. Bogoyavlenskiy A, Berezin V, Prilipov A, Usachev E, Lyapina O, Korotetskiy I, et al. Newcastle disease outbreaks in Kazakhstan and Kyrgyzstan during 1998, 2000, 2001, 2003, 2004, and 2005 were caused by viruses of the genotypes VIIb and VIId. *Virus Genes* (2009) **39**(1):94–101. doi:10.1007/s11262-009-0370-1
21. Bogoyavlenskiy A, Berezin V, Prilipov A, Usachev E, Korotetskiy I, Zaitceva I, et al. Characterization of pigeon paramyxoviruses (Newcastle disease virus) isolated in Kazakhstan in 2005. *Virol Sin* (2012) **27**(2):93–9. doi:10.1007/s12250-012-3234-0
22. Pchelkina IP, Manin TB, Kolosov SN, Starov SK, Andriyasov AV, Chvala IA, et al. Characteristics of pigeon paramyxovirus serotype-1 isolates (PPMV-1) from the Russian Federation from 2001 to 2009. *Avian Dis* (2013) **57**(1):2–7. doi:10.1637/10246-051112-Reg.1
23. Susta L, Hamal KR, Miller PJ, Cardenas-Garcia S, Brown CC, Pedersen JC, et al. Separate evolution of virulent Newcastle disease virus from Mexico and Central America. *J Clin Microbiol* (2014) **52**(5):1382–90. doi:10.1128/JCM.00066-14
24. Kim LM, Afonso CL, Suarez DL. Effect of probe-site mismatches on detection of virulent Newcastle disease viruses using a fusion-gene real-time reverse transcription polymerase chain reaction test. *J Vet Diagn Invest* (2006) **18**(6):519–28. doi:10.1177/104063870601800601
25. Dimitrov KM. *Ethiological and Molecular-Epidemiological Studies of Newcastle Disease in Domestic and Wild Birds*. Sofia: National Diagnostic and Research Veterinary Medicne Institute (2013).
26. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, et al. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *J Clin Microbiol* (2004) **42**(1):329–38. doi:10.1128/JCM.42.1.329-338.2004
27. Stegniy B, Gerilovych A, Bolotin V, Solodiankin O, Muzyka D, Afonso CL. Genetic diversity of velogenic Newcastle disease virus isolates from Ukraine. *16th ICID*. Cape Town (2014).
28. Perozo F, Merino R, Afonso CL, Villegas P, Calderon N. Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. *Avian Dis* (2008) **52**(3):472–9. doi:10.1637/8276-022908-Reg.1
29. Nei M, Kumar S. *Molecular Evolution and Phylogenetics*. New York, NY: Oxford University Press (2000).
30. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* (2013) **30**(12):2725–9. doi:10.1093/molbev/mst197

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 12 October 2015
doi: 10.3389/fpubh.2015.00219

Mapping of networks to detect priority zoonoses in Jordan

*Erin M. Sorrell[1], Mohammad El Azhari[2], Nezar Maswdeh[2], Sarah Kornblet[1], Claire J. Standley[1], Rebecca L. Katz[1], Ibrahim Ablan[2] and Julie E. Fischer[1]**

[1] Milken Institute School of Public Health, George Washington University, Washington, DC, USA, [2] Jordan Field Epidemiology Program, Ministry of Health, Amman, Jordan

Early detection of emerging disease events is a priority focus area for cooperative bioengagement programs. Communication and coordination among national disease surveillance and response networks are essential for timely detection and control of a public health event. Although systematic information sharing between the human and animal health sectors can help stakeholders detect and respond to zoonotic diseases rapidly, resource constraints, and other barriers often prevent efficient cross-sector reporting. The purpose of this research project was to map the laboratory and surveillance networks currently in place for detecting and reporting priority zoonotic diseases in Jordan in order to identify the nodes of communication, coordination, and decision-making where health and veterinary sectors intersect, and to identify priorities and gaps that limit information sharing for action. We selected three zoonotic diseases as case studies: highly pathogenic avian influenza (HPAI) H5N1, rabies, and brucellosis. Through meetings with government agencies and health officials, and desk research, we mapped each system from the index case through response – including both surveillance and laboratory networks, highlighting both areas of strength and those that would benefit from capacity-building resources. Our major findings indicate informal communication exists across sectors; in the event of emergence of one of the priority zoonoses studied, there is effective coordination across the Ministry of Health and Ministry of Agriculture. However, routine formal coordination is lacking. Overall, there is a strong desire and commitment for multi-sectoral coordination in detection and response to zoonoses across public health and veterinary sectors. Our analysis indicates that the networks developed in response to HPAI can and should be leveraged to develop a comprehensive laboratory and surveillance One Health network.

Keywords: one health, zoonotic disease, Jordan, laboratory, disease surveillance

OPEN ACCESS

Edited by:
Jeanne Marie Fair, Defense Threat Reduction Agency, USA

Reviewed by:
Corrie Cabell Brown, University of Georgia, USA
Rebekah Tiller, Centers for Disease Control and Prevention, USA
Shakir Jawad, Uniformed Services University of the Health Sciences, USA

****Correspondence:***
Julie E. Fischer, Department of Health Policy and Management, Milken Institute School of Public Health, George Washington University, 950 New Hampshire Avenue NW, Washington, DC 20052, USA
jfischer@gwu.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *19 June 2015*
Accepted: *16 September 2015*
Published: *12 October 2015*

Citation:
Sorrell EM, El Azhari M, Maswdeh N, Kornblet S, Standley CJ, Katz RL, Ablan I and Fischer JE (2015) Mapping of networks to detect priority zoonoses in Jordan. Front. Public Health 3:219. doi: 10.3389/fpubh.2015.00219

Introduction

The emergence and spread of new pathogens is one of today's highest global health security risks with zoonotic diseases arguably the chief contributor. Zoonoses occur at the interface of human and animal health, impacting a wide range of health services and livelihoods. Social and political issues surround their assessment and management. Zoonotic viruses, parasites, bacteria, and fungi are recognized as threats to human health and sustainable development worldwide, and are a major concern for national and international agencies (1). Significant risk factors for the emergence and

rapid spread of zoonotic diseases include international travel; global trade; increasing interactions among humans, wildlife, exotic, and companion animals; human behavior; rapid microbial adaptation; changing climates and ecosystems; and changing livestock management practices (2). Domestic animals and wildlife are well-known reservoirs of many emerging infectious diseases; roughly 75% of recent emerging infections and 60% of all human pathogens are of zoonotic origin (3–6).

Although zoonotic diseases clearly present a significant threat to human and animal public health, many remain neglected due to competing priorities; for example, ministries of health are coping with growing burdens of non-communicable chronic diseases alongside existing maternal and child health needs, whereas ministries of agriculture/wildlife tend to prioritize livestock management for food production and trade. The costs of zoonoses in lives and livelihoods can be enormous. The effects of zoonoses on human health and economies have recently been underscored by notable outbreaks, such as the 2009 H1N1 influenza virus pandemic, which began in swine farms on the Mexico–US border. Unfounded fears that meat products could transmit "swine flu" led to major losses to the North American pork industry, amounting to 25 million USD per week, and the banning of importation of pigs and pork products by at least 15 countries (7). In addition to natural disease threats, several zoonoses are among agents that have the potential to cause severe health threats if accidentally or deliberately released.

Understanding zoonotic disease emergence, prevention, and control requires multi-disciplinary, collaborative basic and applied research. Communication and coordination among national disease surveillance and response networks are vital in ensuring the timely response to a public health event. Through systematic infectious zoonotic disease data collection, we can gain a better understanding of disease emergence and spread and provide mechanisms upon which to build early warning and response systems for animal and human health. Various frameworks aim to support capacity building for disease surveillance and response, including the World Health Organization's International Health Regulations (IHR), the World Organisation for Animal Health's (OIE) Animal Terrestrial Code and Pathway to Veterinary Services (PVS), and the Global Health Security Agenda (GHSA) (8–11). Although systematic information-sharing between the human and animal health sectors can help decision-makers detect and respond to zoonotic diseases rapidly, resource constraints, and other barriers often prevent efficient cross-sector reporting. Despite significant investments in technology, knowledge, and the availability of the frameworks and programs noted above, many countries still face significant gaps in their abilities to prevent, detect, and respond effectively to public health threats, including zoonotic diseases.

The Hashemite Kingdom of Jordan's abilities to prevent, detect, and respond to zoonoses have been tested and strengthened over recent years, spurred by a large brucellosis outbreak nearly a decade ago and a highly pathogenic avian influenza (HPAI) H5N1 outbreak in 2006. The Ministry of Health's (MOH) Division of Zoonotic Diseases and the Ministry of Agriculture's (MOA) Veterinary Services have developed a strong and cooperative relationship across surveillance and laboratory sectors. Although these relationships exist, they are informal and used only in the context of response to major outbreaks or events. By mapping zoonotic disease detection, reporting, and response capacities across surveillance and laboratory systems, we sought to determine where mechanisms exist to integrate single-disease networks into national zoonotic response and to identify best practices/systems that can be applied across all priority zoonoses. Such mapping not only can help identify hotspots where zoonoses pose significant health threats but also where efforts can be focused to improve prevention, communication, and coordination across veterinary and human health.

Materials and Methods

The methodology consisted of systematically mapping the laboratory and surveillance networks currently in place for detecting and reporting priority zoonotic diseases in Jordan. Our analysis does not include geographical mapping but rather an analysis reviewing major elements of systematic capacity building as outlined by Potter and Brough (12). We identified, collated, and then mapped the current surveillance and laboratory systems in place to detect, assess, report, and respond to zoonotic diseases using publically available reports and key informant interviews. The relevant subject matter experts and other stakeholders for interviews and discussion were selected by the MOH Directorate of Communicable Diseases (DCD) and the MOA Chief Veterinary Officer. We selected three priority zoonotic diseases for our analysis with varying burdens on human and veterinary health sectors to better define nodes of communication and coordination as well as gaps for capacity building and systems strengthening. This type of analysis may identify current vertical, disease-specific strategies and frameworks that can be applied horizontally to develop national zoonotic disease strategies. It is important to note that our mapping does not address the role of livestock keepers and/or the density and number of livestock, which play a major role in disease outbreaks, transmission, and at times subsequent epidemics.

Selection of Priority Zoonoses

There are multiple methods used in prioritizing disease detection and response capacity building, including analysis of the local and national burden of disease; global trends in emergence; economic costs associated and cross-sector impacts; human morbidity and mortality; and population health (3, 13–15). Our goal was to examine coordination and communications from the index case to notification at the national and international levels. In order to determine the mechanisms that promote and/or prevent information sharing across surveillance and laboratory networks both within and among ministries, it was first important to determine the priority zoonoses from both the public and veterinary health sectors. Both MOH and MOA have established priority notifiable disease lists, which are used to strengthen surveillance and laboratory capacities; however, there had not yet been a collaborative discussion on cross-linking these lists to develop formalized multi-sectoral priorities, particularly with respect to zoonotic diseases. We began with reviewing existing MOH and MOA notifiable disease lists and

selecting the zoonotic diseases on each list for consideration. Through collaborative strategic discussions, we identified five MOH–MOA priority zoonoses for further ranking. We selected priority zoonotic diseases for case study analysis that aligned with three major categories of focus for intervention at the animal–human interface: endemic zoonoses, epidemic-prone zoonoses, and emerging zoonoses. Endemic zoonoses account for the majority of human cases and deaths, and the greatest reduction in livestock production. Epidemic-prone zoonoses occur sporadically or cyclically and the spatial distribution of outbreaks may vary, but epidemic-prone diseases are often prioritized due to their impact on health and trade. Emerging zoonoses (diseases that are either new to a population or are rapidly increasing in incidence or geographic range) generally account for only a fraction of the zoonotic disease burden, but outbreaks may have unpredicted and highly disruptive effects (16). We assigned weight to pathogens associated with a high human disease burden (morbidity and mortality); impact on livestock and wildlife (production, economic loss); amenability to practice- or veterinary medicine-based interventions; existing surveillance systems; and, finally, mechanisms for improved stakeholder communication and coordination (17–20).

Mapping of Surveillance and Laboratory Networks

In collaboration with Jordan's Field Epidemiology Training Program (FETP), we developed case studies based on past zoonotic events to examine coordination and communications from the index case to notification at the national and international levels, in order to identify priorities and gaps that limit information sharing for action (**Figure 1**). For the three selected priority zoonoses, we developed case studies outlined in a five-step process: (1) case reporting; (2) reporting and sample submission; (3) laboratory testing; (4) case management; and (5) outbreak investigation (**Figure 2**). For each case study, we created a decision tree at each of the steps noted above, identified the strengths and weaknesses of the system, and recommended steps for improvement. This resulted in a systems map that identified the nodes of communication, coordination, and decision-making where the human and veterinary health sectors intersect, highlighting areas of strength as well as gaps that would benefit from capacity-building resources. This information can be translated into recommendations for strengthening policies, protocols, and practices for preventing and responding to priority zoonoses across veterinary and public health sectors.

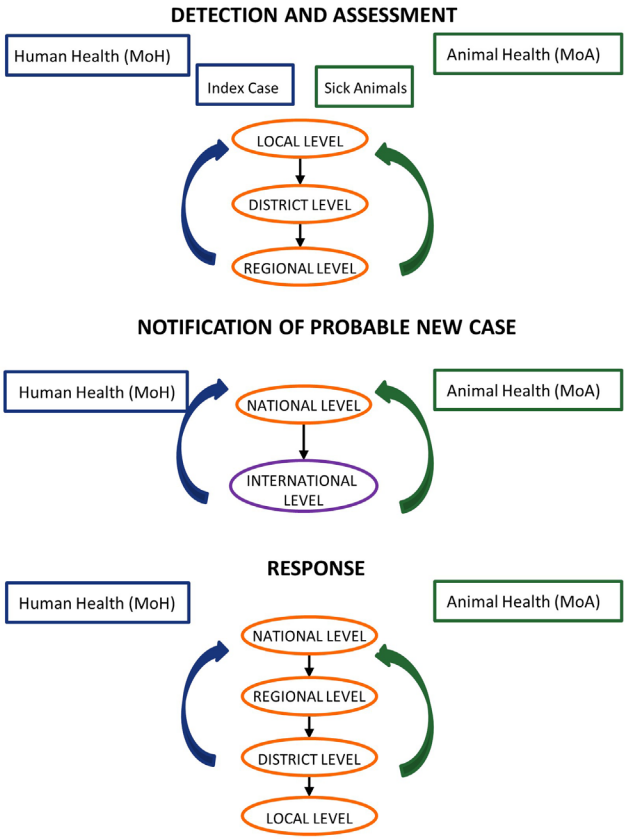

FIGURE 1 | Model joint assessment and response. In collaboration with the Jordan Ministry of Health, Field Epidemiology Training Program, and Ministry of Agriculture, we examined coordination and communications from the index case to notification at the national and international levels in order to identify priorities and gaps that limit information sharing for actions. Efforts in surveillance and response lead by Ministry of Health are represented in blue while those lead by Ministry of Agriculture are in green.

Results

Selecting Priority Zoonoses for Analysis

In collaboration with the MOH-DCD and the MOA Veterinary Services, the combined Jordan FETP and The George Washington University Global Health Security Program (GWU) research team determined that the most suitable priority diseases for our analysis included HPAI H5N1, brucellosis, and rabies. These priority diseases represent endemic zoonoses (brucellosis), epidemic-prone zoonoses (rabies, defined as a disease in which exposures to a single infected animal can lead to multiple human cases) (16), and emerging zoonoses (HPAI H5N1).

Mapping Surveillance Networks

The Ministry of Health is the largest financer and provider of health services in Jordan. Disease surveillance efforts in Jordan fall under the oversight of the Director of Primary Health Care Administration, which oversees eight directorates within MOH (21). The DCD within the Primary Health Care Administration is charged with disease surveillance and is most active in detection, surveillance, assessment, response, and reporting activities. Within DCD, the Surveillance Department, Division of Applied Epidemiology, and Division of Infection Control (among others) oversee specific programs and functions. DCD's Surveillance Department receives and manages information from 22 surveillance sites throughout Jordan that track the 42 reportable diseases in country. Information flows from the health facility level to the health directorates, and then to DCD, where data are compiled and analyzed to prepare the weekly reporting bulletin. Within

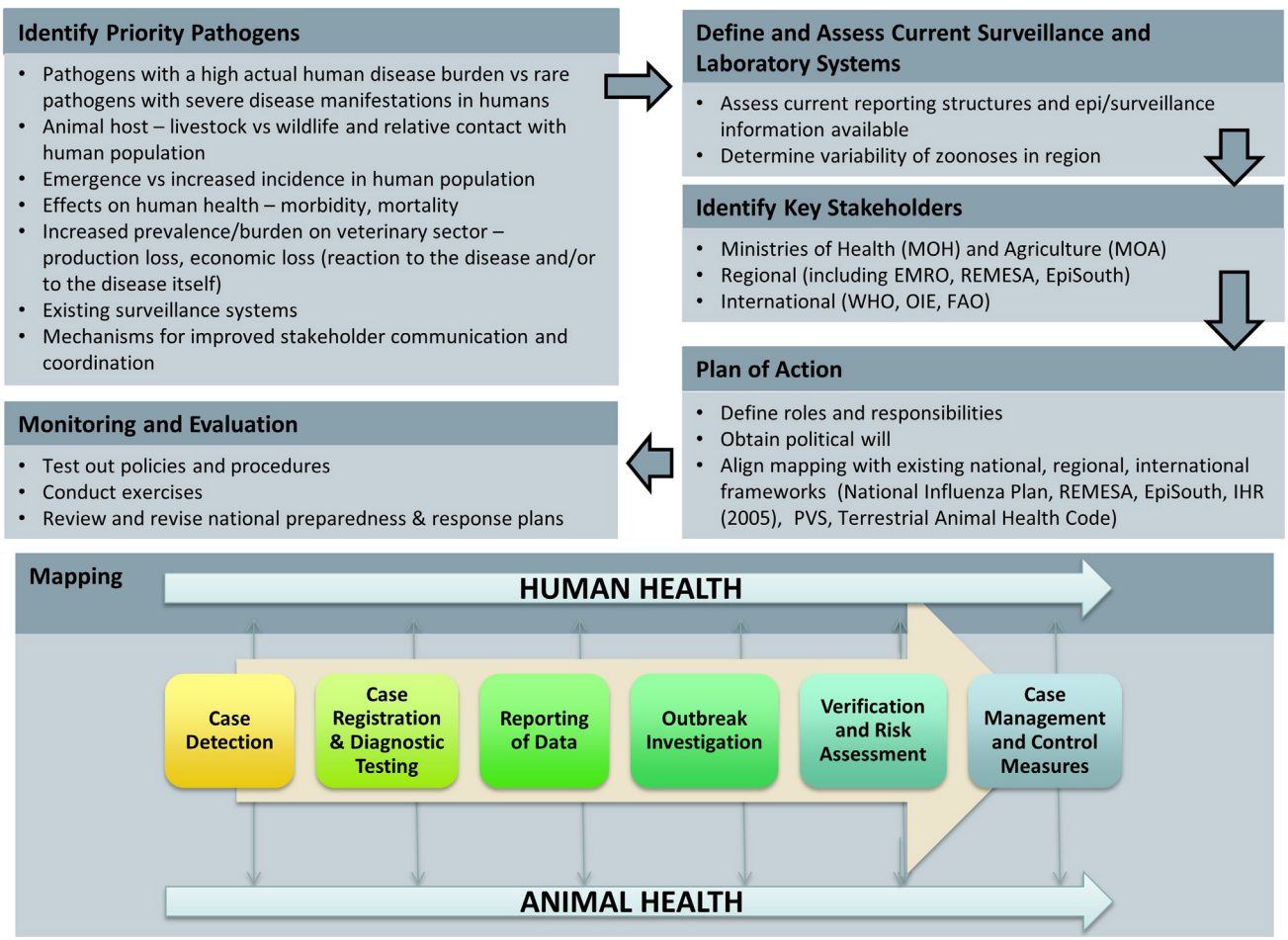

FIGURE 2 | Identifying priority zoonoses and identification and networks for case management. In order to select three priority zoonotic diseases for analysis, we considered MOH and MOA notifiable disease lists as well as global priority zoonoses. For the three selected priority zoonoses, we developed case studies outlined in a five-step process: (1) case reporting; (2) reporting and sample submission; (3) laboratory testing; (4) case management; and (5) outbreak investigation. The resulting analysis is a systems map that identified the nodes of communication, coordination, and decision-making where the health and veterinary sectors intersect, highlighting both areas of strength and gaps that would benefit from capacity-building resources.

MOA, the Secretary General Assistant for Livestock and the Chief Veterinary Officer have responsibility for the organization and implementation of veterinary services, whereas the majority of administrative control falls to 13 agricultural departments. Veterinarians are trained in the field on zoonoses communication and reporting, sample collection, and packaging. Within the Veterinary Services Department, there is an Animal Health Section, Poultry Health Section, and Veterinary Quarantine Section, which coordinate with the governorate level departments on disease surveillance and response. Both MOH and MOA have notifiable disease lists for immediate, weekly, and monthly reporting.

Mapping Laboratory Networks

Diagnostic and confirmatory laboratory services are provided from the Central Public Health Laboratory (CPHL) to the health center level. CPHL oversees laboratory biosafety and biosecurity programs for MOH laboratories and hospitals. Each health directorate has a laboratory coordinator at the governorate level. Although Laboratory Quality Management Systems (LQMS) and the logistical support to manage supplies and safe specimen transport exist they are uneven at the subnational level. Diagnostic and confirmatory testing capabilities are shared across public and private sector laboratories, which can provide challenges in the event of major outbreaks. CPHL coordinates with the U.S. Naval Medical Research Unit 3 (NAMRU-3) located in Cairo for confirmatory testing when necessary. MOA has veterinary laboratories in each of the 12 governorates that perform routine diagnostics at varying levels of capacity. A lack of resources, both human and financial, leads to a majority of diagnostic and confirmatory testing falling to the Central Veterinary Laboratory (CVL) (22). MOA coordinates with the UN Food and Animal Organization (FAO) and OIE to assist with confirmatory testing, as well as gold standard diagnostic tests when these are not locally available.

Case Study #1: Highly Pathogenic Avian Influenza H5N1

As of 2006, Jordan and most of its neighbors have remained free of human HPAI H5N1 cases, with the exception of Egypt (which reported 48 deaths and 165 cases between November 2014 and April 2015) (23–25). Jordan's geography puts it at low risk for the introduction of HPAI from migratory waterfowl due to its lack of surface water; key migratory bird habitats in the Jordan Valley and around the Gulf of Aqaba are distant from major poultry production facilities. A majority of Jordan's poultry farms are commercial with backyard flocks comprising only 2% of the sector (22). The commercial sector is advanced for the region, including biosecurity into its best practices (26).

Existing Networks

Following devastating outbreaks of HPAI H5N1 in 2006, Jordan established the National Committees on Avian and Pandemic Influenza, including the National Steering Committee, the National Technical Committee, and the National Center for Security and Crisis Management (previously the Disaster Management Committee) each playing a role in detection, reporting, and response to highly pathogenic and pandemic influenza. Jordan has both an Animal Health National Preparedness Plan and National Contingency Plan for Avian Influenza, which are utilized by various ministries, including Ministries of Health, Agriculture, Planning, Foreign Affairs, Transport and Communication, Interior, Industry and Trade, Education, Communications and IT among others. At the regional level, the Middle East Consortium on Infectious Disease Surveillance (MECIDS) network developed an Avian and Pandemic Influenza Sub-Regional Common Plan of Action for Palestine, Jordan, and Israel[1]. The plan defines the protection zone (3 km radius from affected farm designated for culling), surveillance zone (10 km radius from affected farm where enhanced surveillance and control measures must be taken), and case definitions for avian and human influenza cases (suspected, probable, and confirmed). It also outlines principles, procedures, and protocols for MOA and MOH officials in the case of H5N1 in poultry (notification of suspected case, protection and surveillance zone established, lab confirmation of H5, follow-up) and in the event of H5N1 in humans (notification of suspected case, epidemiological investigation, lab diagnosis of H5 and follow-up). In 2008, 32 representatives from multiple sectors (health, transportation, education, interior, laboratory, and media) in Jordan, Palestine, and Israel participated in a regional pandemic influenza tabletop exercise to develop action items based on various influenza case scenarios, including human-to-human transmission of HPAI H5N1. This body is active in disease surveillance and response across a number of priority diseases for the region and is able to activate and respond in the event of HPAI if necessary. The 2006 HPAI H5N1 outbreak in poultry is a good example of how and when MOH and MOA communicate, particularly when there was an immediate need and financial resources.

[1]http://www.mecidsnetwork.org/

Detection, Notification, and Response

If a patient presents at a health facility or hospital and the clinician suspects HPAI based on clinical symptoms or due to reports of contact with sick poultry, the patient is isolated and samples are sent to the CPHL for diagnostic confirmation. The isolated patient is treated with antivirals and health care staff involved in patient care receives preventative treatment. HPAI is an immediate reportable Group A disease; the primary health care unit or hospital reports directly to the Health Directorate, which then reports to the DCD. MOH also communicates with MOA that there is a suspect human case of HPAI. Likewise, if there are reports of poultry deaths and/or an animal presents and is characterized as suspect HPAI, veterinary services will notify MOA, MOH, and collect samples for confirmation testing at the CVL. A positive rapid diagnostic test for type A influenza may result in quarantine or culling of affected farms while confirmation testing is performed at CVL. MOA reports positive cases to OIE on a monthly basis, whereas MOH would immediately report a positive human case as outlined under IHR (2005). If the CVL confirms HPAI, Rapid Response Teams (RRTs) assist in providing personal protective equipment (PPE) and restricting contact to affected farms/flocks to determine proper culling procedures. In addition, a poultry vaccination team will be deployed to farms/flocks within a 7-km radius. In the event of a confirmed human case public health RRTs will conduct in-depth reports and follow-up with possible suspect cases and contacts. If the patient's symptoms persist with unconfirmed diagnosis, treatment with Tamiflu continues for 7 days and care is provided per physician recommendations. During an outbreak MOH and MOA will communicate laboratory confirmed cases to each other on a daily basis. Jordan has both an Animal Health National Preparedness Plan and National Contingency Plan for Avian Influenza, which are utilized by various ministries including MOA and MOH. **Figure 3** depicts a flow chart schematic of surveillance and laboratory channels. Mechanisms for communication and coordination among laboratory, public health, and veterinary officials at the governorate and national level are strong in the event of a suspect case of HPAI H5N1. Frameworks and plans exist and function well; however, they are only activated in the case of emergencies.

Case Study #2: Rabies

Rabies is a zoonotic viral disease that causes acute inflammation of the brain in animals. The disease is spread to humans from another animal (e.g., dogs, camels, donkeys), commonly by a bite or scratch, although exposure of mucous membranes to infected saliva is also a risk. Globally, most cases are the result of a dog bite: exposure to rabid dogs is the cause of over 90% of human exposures to rabies and of over 99% of human deaths worldwide. Rabies is a completely preventable disease in the human population with effective veterinary vaccine campaigns and effective reporting and rapid post-exposure treatment following animal bites. More than 50,000 people die annually from rabies worldwide, despite the fact that the tools to prevent and manage the disease are readily available (27). Once clinical signs of rabies appear, the disease is nearly always fatal, and treatment is typically supportive.

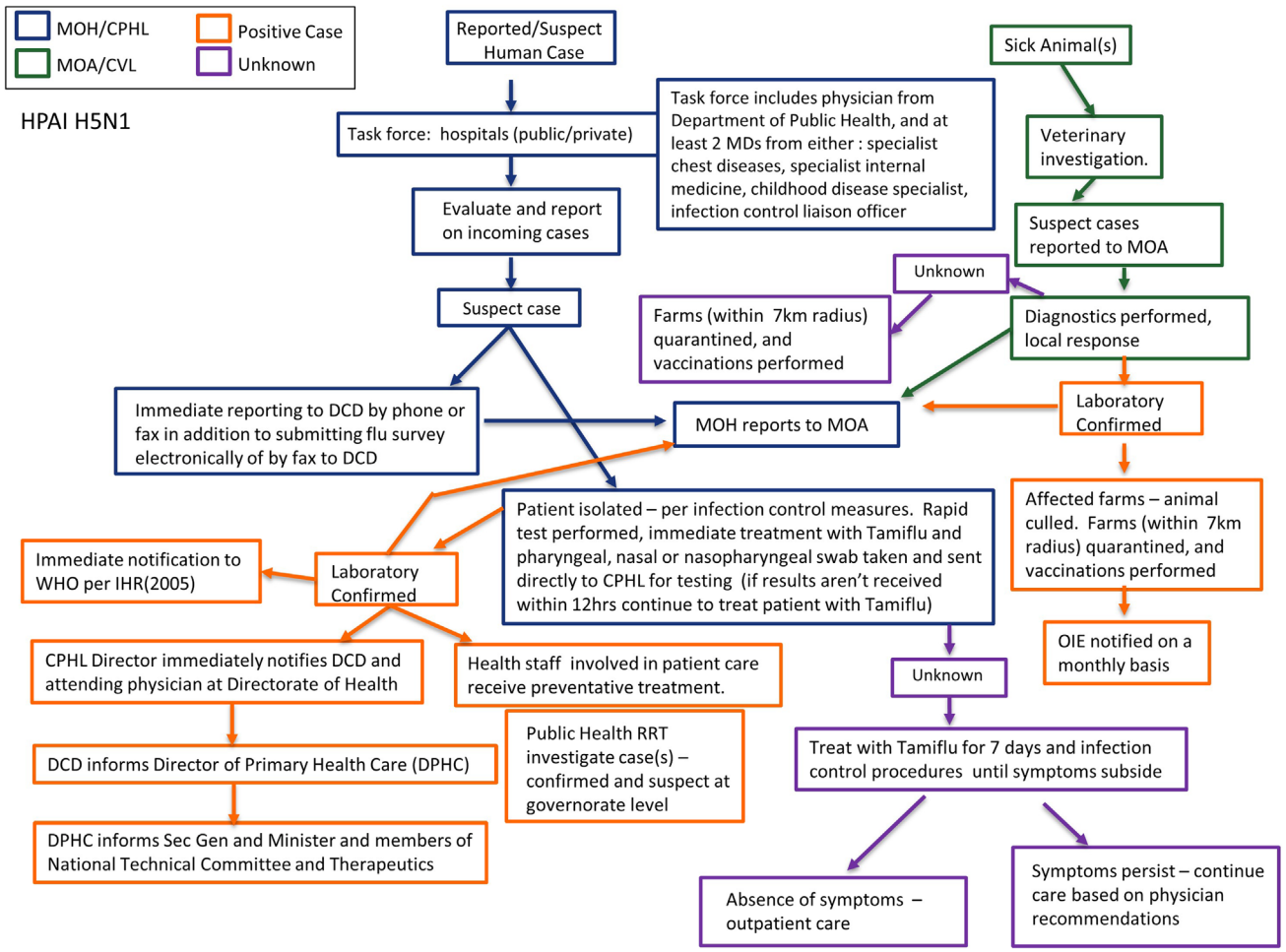

FIGURE 3 | Surveillance and laboratory mapping of highly pathogenic avian influenza (HPAI) H5N1. Surveillance and laboratory networks for HPAI H5N1, including nodes of communication and response, were mapped across human health and veterinary sectors. Systems were analyzed beginning from report of a suspect case to diagnostic confirmation, including evaluation: case reporting; reporting and sample submission; laboratory testing; case management; and outbreak investigation. Efforts in surveillance and response lead by Ministry of Health are represented in blue while those lead by Ministry of Agriculture are in green. Positive cases are noted in orange while unknown/unconfirmed cases are represented in purple. Abbreviations: MOH, Ministry of Health; CPHL, Central Public Health Laboratory; DCD, Directorate of Communicable Diseases; MOA, Ministry of Agriculture; CVL, Central Veterinary Laboratory.

Existing Networks

Human rabies cases are rather rare in Jordan. Dog bites account for the vast majority of suspect human rabies cases in Jordan (28). According to MOH, 4753 patients were treated for rabies exposure in 2013, but no human rabies cases were reported (or have been for the last 3 years). MOA reported a total of seven cases and seven deaths to OIE in 2013 (29). MOA provides free vaccines to vaccinate animals for prevention and control of rabies; however, there is a limited vaccine supply and an inability to cover the entire susceptible population. Currently, vaccine campaigns focus on the companion animal population, covering stray dogs only as supplies allow. There is no policy to vaccinate any potential wildlife reservoir. Key to the control of rabies in Jordan is the containment and vaccination of the stray dog population nationwide.

Detection, Notification, and Response

Any human bitten by stray or wild dogs is considered a probable rabies case and the responsible health official uses the case definition for determination. All suspect patients are treated post-exposure with the rabies vaccination and MOH covers all costs for post-exposure prophylaxis and supportive care. Patient samples are collected and sent to the Department of Sera and Vaccines for confirmation, however, testing of samples is not routine, which can lead to unnecessary costs of patient care from post-exposure prophylaxis for unconfirmed rabies cases. Rabies is an immediately notifiable disease, MOH notifies MOA of suspect cases; however, animal bites are reported to MOA on a weekly basis and to OIE annually. The Surveillance Unit within MOH conducts investigations into suspect cases and submit final reports to DCD. Occasionally, the RRT includes

veterinarians and subject matter experts from MOA. In the event of suspect rabies case(s) in domesticated or wild animals, the local veterinary services is notified and if based on case definition the animal is labeled suspect, MOA is immediately notified for investigation. If the suspect case is in feral or otherwise non-domesticated animal(s), they are immediately culled without quarantine. If the animal(s) are domesticated, they are quarantined for 10 days under the observation of MOA; if the animal develops symptoms or succumbs to infection, samples are sent to MOH-Department of Sera and Vaccines for diagnostic confirmation. There is currently no public veterinary laboratory in Jordan that has capacity to diagnose rabies in animals. MOA will conduct an investigation of neighboring areas for additional cases and quarantine when necessary. **Figure 4** shows a flow chart schematic of surveillance and laboratory channels. Key to the control of rabies in Jordan is the containment and vaccination of the stray dog population nationwide and timely confirmation of suspect human cases in order to prevent unnecessary extensive health care costs for post-exposure treatments on negative patients.

Case Study #3: Brucellosis

Brucellosis is an important zoonotic disease of livestock, notifiable to OIE (30). Globally, human brucellosis is a re-emerging zoonotic disease with an estimated 2% case fatality rate, even though successful eradication and control programs for domestic animals effectively and significantly decrease disease incidence in humans, and have been established in many at-risk countries. Symptoms of brucellosis in humans include fever with multiple non-specific clinical signs and symptoms. Delayed diagnosis, chronic disease, failure of primary antibiotic treatment, and relapses are common. Brucellosis is transmitted through exposure to infected animal products (most commonly raw dairy products) or, less frequently, through direct contact

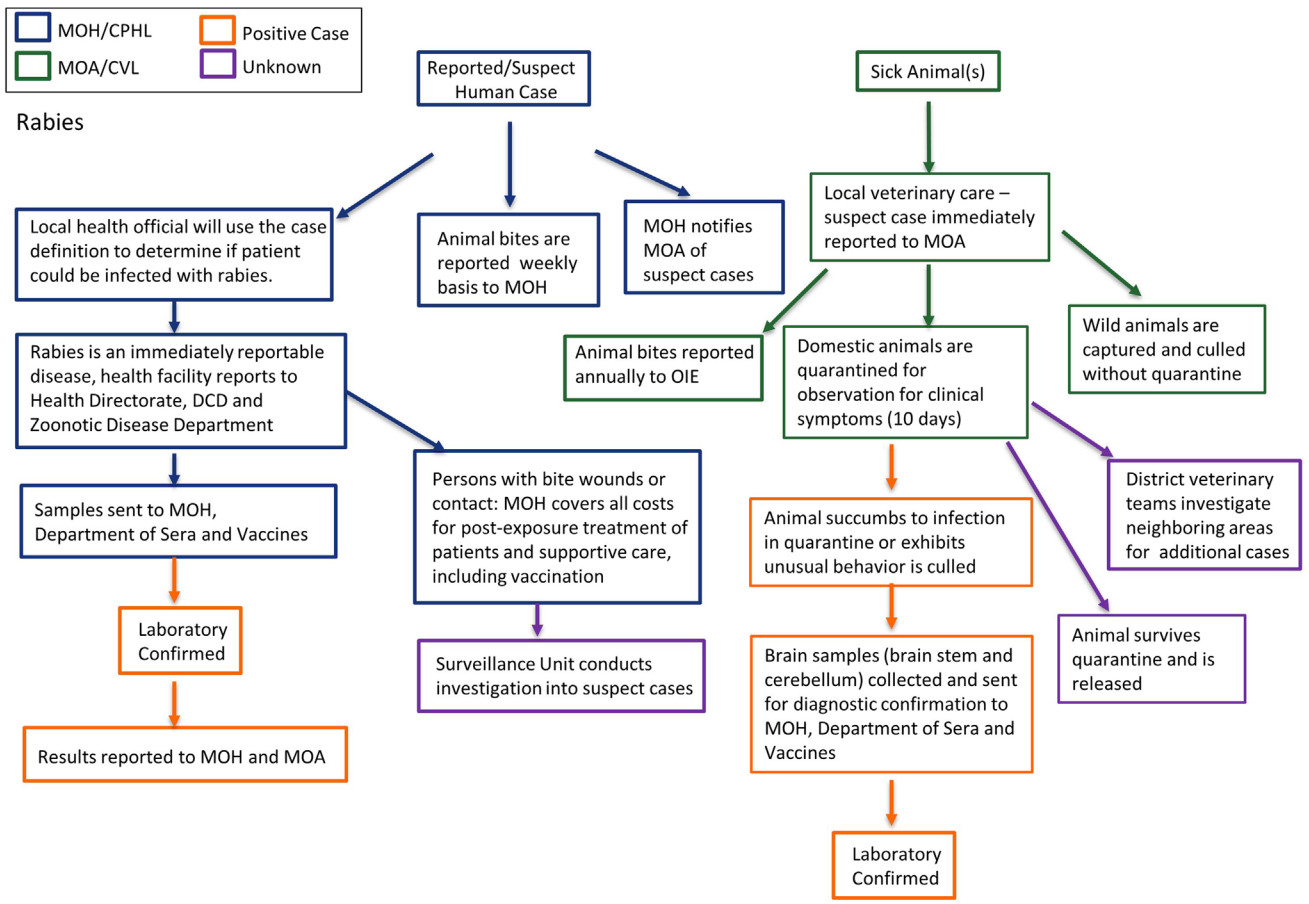

FIGURE 4 | Surveillance and laboratory mapping of rabies. Surveillance and laboratory networks for rabies, including nodes of communication and response, were mapped across human health and veterinary sectors. Systems were analyzed beginning from report of a suspect case to diagnostic confirmation, including evaluation: case reporting; reporting and sample submission; laboratory testing; case management; and outbreak investigation. Efforts in surveillance and response lead by Ministry of Health are represented in blue while those lead by Ministry of Agriculture are in green. Positive cases are noted in orange while unknown/unconfirmed cases are represented in purple. Abbreviations: MOH, Ministry of Health; CPHL, Central Public Health Laboratory; DCD, Directorate of Communicable Diseases; MOA, Ministry of Agriculture; CVL, Central Veterinary Laboratory.

with infected camels, cattle, sheep, or goats. More than 500,000 human cases are reported worldwide each year, (31) but the number of undetected cases is believed to be considerably higher. *Brucella* spp. are also categorized as potential biological agents for deliberate use in many US and international frameworks due to their high contagiousness and their impact on human and animal health.

Existing Networks

In 1985, an official system for reporting human cases of brucellosis was established by MOH, under the supervision of the Communicable Diseases Control Program Division. Spurred by a large brucellosis outbreak in Jordan roughly 10 years ago, the MOH's Division of Zoonotic Diseases and veterinary public health actors at the MOA developed a cooperative relationship in reporting and response to brucellosis. However, there is no national plan. According to OIE reporting, brucellosis continues to be in the top three zoonotic diseases reported in Jordan (29). In collaboration with CDC, the MOH and others conducted a burden of illness study in 2003, including population, animal vaccinations, and laboratory surveys and validation study. However, outbreaks are still prevalent in Ma'an and Mafraq governorates on a seasonal basis and for various reasons, including the lack of clear clinical symptoms and misdiagnosis, human brucellosis is significantly under-reported and underdiagnosed, particularly by the private health sector (32, 33). In Jordan, ruminants, particularly sheep and goats, are vaccinated at all ages, at any time during the year, and annual revaccination is recommended. On average, about 18–25% of the sheep and goats in Jordan were vaccinated through 2000, although unofficial estimations on vaccine coverage is increasing and can be estimated at times to be as high as 50% recently published data indicates that only 1.5% of the small ruminant population is vaccinated leading to regional endemicity, particularly in the north (34, 35). Starting in 2015 a new project will begin, a partnership between EMPHNET and CDC with Jordan MOH as lead implementer, to estimate disease burden in the human population (36).

Detection, Notification, and Response

When a patient presents with symptoms consistent with brucellosis and has ingested raw milk or other potentially infected dairy products, the health official will use the case definition to determine whether to classify the case as suspect brucellosis. Suspect human cases are reported to MOH and the Occupational Health and the Food and Drug Agency of Jordan. Patients may be admitted to a fever hospital to confirm diagnosis and initiate treatment. Clinical samples are sent to the governorate level laboratory for initial diagnostic testing, and to CPHL for confirmatory testing as indicated. The lab results are not shared with MOA. When possible, health education is provided to at-risk occupational groups (farmers, meat packers, dairies) working with animals or animal products; however, there is no clear guidance for surveillance and outbreak response for MOH. In the event of a suspect case or farm(s), the local veterinary services will quarantine the suspect farm(s) and collect samples for diagnostic testing at the CVL, at times and when possible governorate level labs will perform diagnostics. A team is sent to each suspect farm to conduct an investigation, which includes an imposed quarantine, provision of herd vaccination history, sample collection, and testing. A farm must test negative three consecutive times before being cleared. Any animals testing positive must be culled. It should be noted that this is the recommended procedure; however, we do not have country-wide data as to whether this is implemented. MOA reports all positive cases to OIE. Individual animal cases of brucellosis are not reported to MOH due to the endemicity of brucellosis in Jordan; however, outbreaks are reported directly to DCD. Please see **Figure 5** for a flow chart schematic of surveillance and laboratory channels. As noted above, effective livestock vaccine campaigns can significantly reduce the burden of human brucellosis. There are clear seasonal patterns associated with human cases and outreach and education on zoonotic transmission will be key in containing human outbreaks.

Discussion

Mapping of zoonoses and the burden of such diseases can help identify vulnerabilities not only where zoonoses pose significant health threats but also where efforts can be focused to improve prevention, communication, and coordination across veterinary and human health. These study findings describe existing systems that can be strengthened or applied by stakeholders to address current needs within Jordan, and offer case studies that can be applied in other contexts. Although the findings may appear predictable to those already deeply familiar with Jordan's surveillance and response systems, the formal linkages within and across sectors may not be immediately obvious to the increasingly diverse stakeholder and partner networks engaged in long-term capacity building for global health security.

We found many similarities in surveillance and response capacities across local, governorate, and national public and veterinary health networks regardless of the pathogen mapped, indicating that improvement in response to one specific pathogen would most likely improve the ability to respond to other zoonoses (**Figure 6**). The results of our mapping highlighted three main areas for improvement toward building national One Health capacities: (1) a national zoonotic reporting and communication framework, (2) a national zoonotic preparedness and response plan, and (3) increased laboratory diagnostic capacity across governorate level laboratories.

National Zoonotic Reporting and Communication Framework

There are strong informal mechanisms for communication and coordination within and across local public health and veterinary services with consistent reporting up to governorate and national levels. However, the local facilities are not always involved in outreach and communication strategies for local response. There is no standardized structure for communication and information

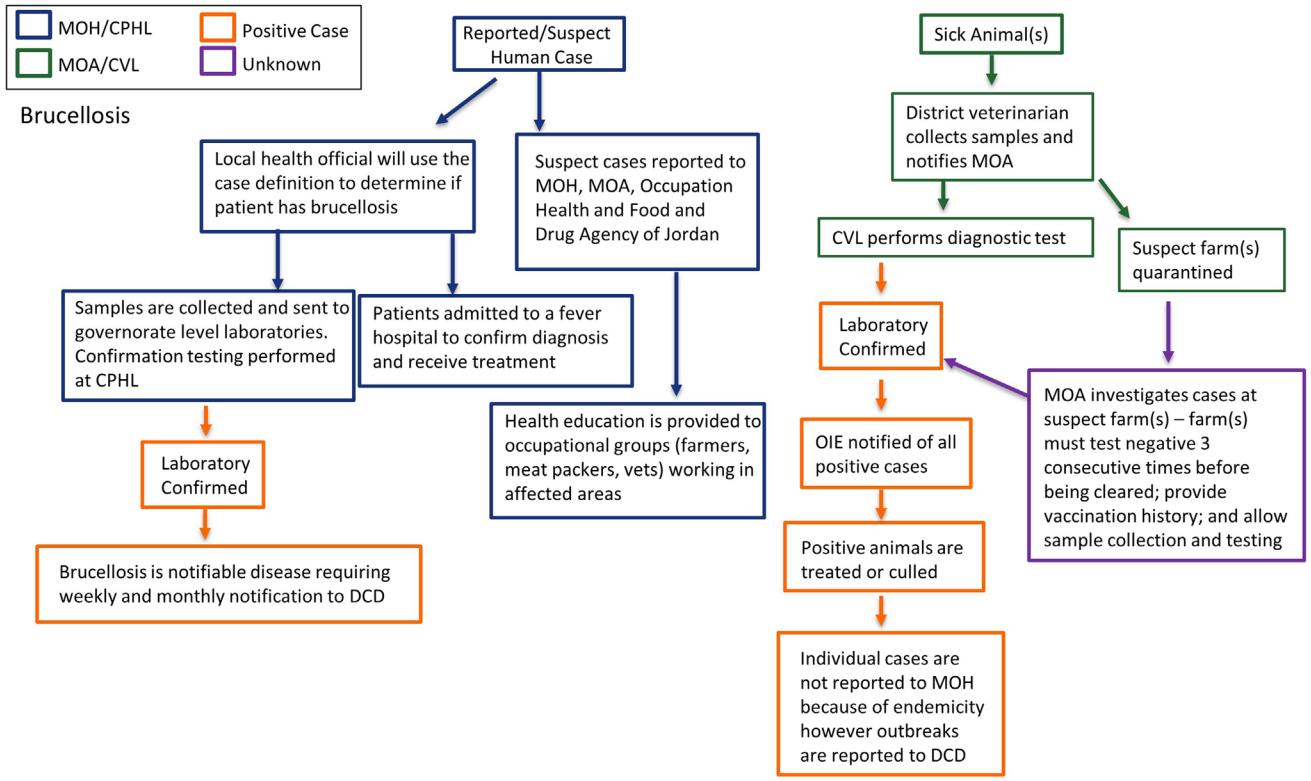

FIGURE 5 | Surveillance and laboratory mapping of brucellosis. Surveillance and laboratory networks for brucellosis, including nodes of communication and response, were mapped across human health and veterinary sectors. Systems were analyzed beginning from report of a suspect case to diagnostic confirmation, including evaluation: case reporting; reporting and sample submission; laboratory testing; case management; and outbreak investigation. Efforts in surveillance and response lead by Ministry of Health are represented in blue while those lead by Ministry of Agriculture are in green. Positive cases are noted in orange while unknown/unconfirmed cases are represented in purple. Abbreviations: MOH, Ministry of Health; CPHL, Central Public Health Laboratory; DCD, Directorate of Communicable Diseases; MOA, Ministry of Agriculture; CVL, Central Veterinary Laboratory.

sharing across and within surveillance sectors and laboratories. There is no formal mechanism or protocol for reporting laboratory confirmation beyond CPHL and CVL obligations to report back to their relevant ministry departments. There is little, if any, cross-talk between CPHL and CVL in both surveillance reports and laboratory confirmation. This node of cross-sector communication is of particular importance when considering sentinel and early warning systems for zoonotic disease outbreaks in the veterinary sector and in investigations and response during simultaneous outbreaks of zoonoses in humans and animals. We recommend establishing a framework for reporting and communication to and from ministry department focal points to their local and governorate counterparts as well as across sectors at each level of reporting.

National Zoonotic Preparedness and Response Plans

Rapid response teams, both locally and nationally deployed, are effective in outbreak investigation within their respective sectors; however, organization and deployment of multi-disciplinary RRTs are extremely pathogen dependent. This inconsistency can lead to duplication of efforts during critical phases of outbreak response and containment. Although there are disease-specific plans, such as the Animal Health National Preparedness Plan and National Contingency Plan for Avian Influenza, no national framework for preparedness and response to priority zoonotic diseases exists. We recommend that RRTs should be multi-disciplinary at the national level, using the FETP as resource to link governorate level epidemiologists available for rapid response.

Laboratory Capacity

Local and governorate level public health and veterinary laboratory capacity is inconsistent. Some labs lack the ability to perform routine diagnostics, due either to constraints in infrastructure, equipment, human resources, and/or funding. This inconsistency leads to delays in time to pathogen confirmation and response as well as increased diagnostic burdens on the national level laboratories, and at times, outsourcing to private laboratories for diagnostic confirmation. We propose that Jordan develop a national laboratory network, modeled after their experience as a member of the Network for the Control of Public Health Threats

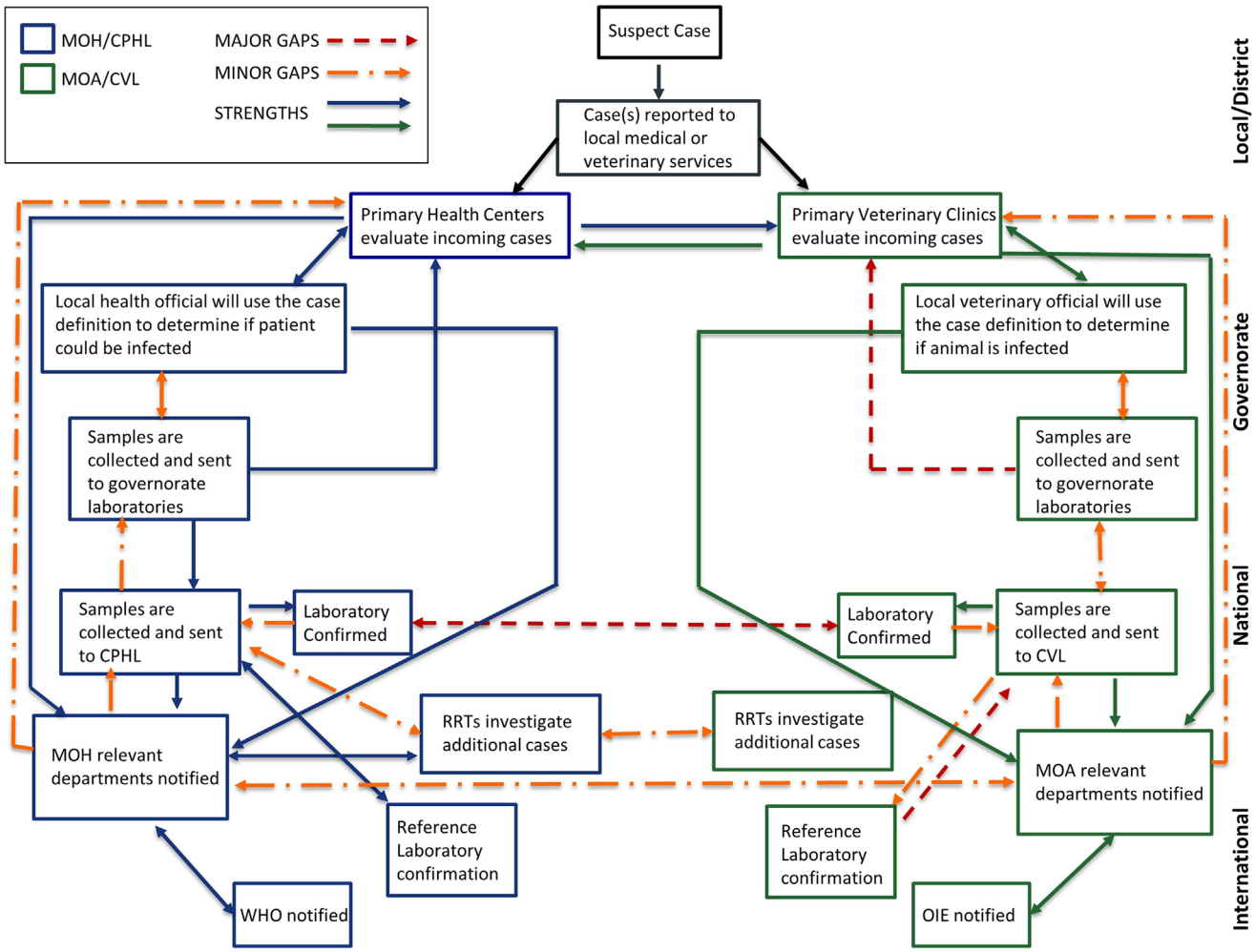

FIGURE 6 | Mapping public health and veterinary surveillance and laboratory networks. An overall analysis of existing surveillance and laboratory networks for zoonotic diseases, including nodes of communication and response, were mapped across human health and veterinary sectors to indicate areas of strength and those requiring strengthening. Efforts in surveillance and response lead by Ministry of Health are represented in blue while those lead by Ministry of Agriculture are in green. Strengths within and across sectors are represented by solid blue and green lines. Major gaps are represented in red while minor gaps are represented in orange. Abbreviations: MOH, Ministry of Health; CPHL, Central Public Health Laboratory; DCD, Directorate of Communicable Diseases; MOA, Ministry of Agriculture; WHO, World Health Organization; OIE, World Organisation for Animal Health; RRTs, Rapid Response Teams.

in the Mediterranean Regional and South East Europe (EpiSouth) Laboratory Network, to provide a formalized, standard protocol for private and public laboratory partnership for diagnostic testing or priority pathogens in the event of public and veterinary health events and those for routine testing for sentinel surveillance efforts.

Although this project focused on three priority zoonotic diseases in Jordan the challenges identified from both public health and veterinary surveillance and laboratory sectors are challenges faced by many middle income countries. Our analysis indicates that the HPAI networks in Jordan are well developed, coordinated, and effective in event identification, diagnosis, and response, which suggests that these existing resources can and should be leveraged to develop a comprehensive laboratory and surveillance One Health network.

Author Contributions

ES, SK, JF, and RK conceived of the research; MA, NM, SK, CS, ES, and IB conducted the research; MA, NM, SK, ES, IB, and JF wrote the manuscript; CS and RK provided technical review and input to the manuscript.

Acknowledgments

The authors would like to thank Dr. Rachel Dodeen from the Ministry of Agriculture, Zoonotic Disease Team and Andrea Vaught from George Washington University, for their technical review and input. This research collaboration was funded through CRDF Global and the U.S. Department of State Biosecurity Engagement Program, CRDF_BEP-23036.

References

1. Marano N, Pappaioanou M. Historical, new, and reemerging links between human and animal health. *Emerg Infect Dis* (2004) **10**(12):2065–6. doi:10.3201/eid1012.041037
2. Smolinski MS, Hamburg, MA, Lederberg J, editors. *Microbial Threats to Health: Emergence, Detection, and Response*. Washington, DC: The National Academies Press (2003).
3. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. *Nature* (2008) **451**(7181):990–3. doi:10.1038/nature06536
4. Taylor LH, Latham SM, Woolhouse ME. Risk factors for human disease emergence. *Philos Trans R Soc Lond B Biol Sci* (2001) **356**(1411):983–9. doi:10.1098/rstb.2001.0888
5. Woolhouse ME, Gowtage-Sequeria S. Host range and emerging and reemerging pathogens. *Emerg Infect Dis* (2005) **11**(12):1842–7. doi:10.3201/eid1112.050997
6. Grace D, Gilbert J, Randolph T, Kang'ethe E. The multiple burdens of zoonotic disease and an Ecohealth approach to their assessment. *Trop Anim Health Prod* (2012) **44**(Suppl 1):S67–73. doi:10.1007/s11250-012-0209-y
7. Keusch GT, Pappaioanou M, Gonzalez MC, Scott KA, Tsai P, editors. *Sustaining Global Surveillance and Response to Emerging Zoonotic Diseases*. Washington, DC: National Research Council (US) Committee on Achieving Sustainable Global Capacity for Surveillance and Response to Emerging Diseases of Zoonotic Origin (2009).
8. GlobalHealth.Gov. *The Global Health Security Agenda (2015) United States Department of Health and Human Services*. Available from: http://www.globalhealth.gov/global-health-topics/global-health-security/ghsagenda.html
9. World Health Organization. *International Health Regulations (2005)*. 2nd ed (2008). Available from: http://www.who.int/ihr/9789241596664/en/
10. World Organisation for Animal Health. *OIE Tool for the Evaluation of Performance of Veterinary Services (2013)*. Paris: World Organisation for Animal Health. Available from: http://www.oie.int/fileadmin/Home/eng/Support_to_OIE_Members/pdf/A_PVS_Tool_Final_Edition_2013.pdf
11. World Organisation for Animal Health. *Terrestrial Animal Health Code (2014)*. 23rd ed Paris: World Organisation for Animal Health (2014).
12. Potter C, Brough R. Systemic capacity building: a hierarchy of needs. *Health Policy Plan* (2004) **19**(5):336–45. doi:10.1093/heapol/czh038
13. Murray CJ, Lopez AD. Measuring the global burden of disease. *N Engl J Med* (2013) **369**(5):448–57. doi:10.1056/NEJMra1201534
14. Narrod C, Zinsstag J, Tiongco M. A one health framework for estimating the economic costs of zoonotic diseases on society. *Ecohealth* (2012) **9**(2):150–62. doi:10.1007/s10393-012-0747-9
15. Jordan H, Dunt D, Hollingsworth B, Firestone SM, Burgman M. Costing the morbidity and mortality consequences of zoonoses using health-adjusted life years. *Transbound Emerg Dis* (2014). doi:10.1111/tbed.12305
16. Grace D, Mutua F, Ochungo P, Kruska R, Jones K, Brierly L, et al. *Mapping of Poverty and Likely Zoonoses Hotspots*. Nairobi (2012).
17. Perry B, Grace D. The impacts of livestock diseases and their control on growth and development processes that are pro-poor. *Philos Trans R Soc Lond B Biol Sci* (2009) **364**(1530):2643–55. doi:10.1098/rstb.2009.0097
18. Dufour B, Moutou F, Hattenberger AM, Rodhain F. Global change: impact, management, risk approach and health measures – the case of Europe. *Rev Sci Tech* (2008) **27**(2):529–50.
19. Havelaar AH, van Rosse F, Bucura C, Toetenel MA, Haagsma JA, Kurowicka D, et al. Prioritizing emerging zoonoses in the Netherlands. *PLoS One* (2010) **5**(11):e13965. doi:10.1371/journal.pone.0013965
20. Cardoen S, Van Huffel X, Berkvens D, Quoilin S, Ducoffre G, Saegerman C, et al. Evidence-based semiquantitative methodology for prioritization of foodborne zoonoses. *Foodborne Pathog Dis* (2009) **6**(9):1083–96. doi:10.1089/fpd.2009.0291
21. Jordan Ministry of Health. *Ministry of Health Annual Statistical Book 2011*. Amman: Government of Jordan (2013). Available from: http://apps.moh.gov.jo/MOH/Files/Publication/PDF2011_1.pdf
22. Sanchez MP, Schaeffer J. *National Assessment of Avian and Pandemic Influenza Preparedness – Kingdom of Jordan*. Development Alternatives, Inc (DAI) (2007).
23. WHO Regional Office for the Eastern Mediterranean. *Egypt: Upsurge in H5N1 Human and Poultry Cases but no Change in Transmission Pattern of Infection (2015)*. World Health Organization. Available from: http://www.emro.who.int/egy/egypt-news/upsurge-h5n1-human-poultry-cases-may-2015.html
24. World Health Organization. *Cumulative number of Confirmed Human Cases for Avian Influenza A (H5N1) Reported to WHO, 2003-2015*. WHO/GIP (2015). Available from: http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/
25. Al-Natour MQ, Abo-Shehada MN. H5N1 influenza outbreak during March 2006 in Jordan. *Sci Res* (2012) **4**(12):1371–9. doi:10.4236/health.2012.412199
26. Food and Agriculture Organization of the United Nations. *Approaches to Controlling, Preventing and Eliminating H5N1 Highly Pathogenic Avian Influenza in Endemic Countries*. Rome: Animal Production and Health Paper. No. 171 (2011). Available from: http://www.fao.org/docrep/014/i2150e/i2150e.pdf
27. World Health Organization. *Rabies*. Geneva: World Health Organization (2015). Available from: http://www.who.int/rabies/en/
28. Haddadin RHS, Khazally H, Mhedat A, Al-Rashdan M, Al-Nsour M, Al-Hajawi B. Animal bites and animal rabies surveillance, Jordan, 2000–2007. *Poster presented at Journées de veille sanitaire*; 2008 Nov 26–28; Paris. Available from: http://www.invs.sante.fr/publications/2008/jvs_2008/22_drrajaa_poster.pdf
29. World Animal Health Information Database (WAHID). *Version 1. World Organisation for Animal Health (OIE)* (2015). Available from: http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home
30. Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis* (1997) **3**(2):213–21. doi:10.3201/eid0302.970219
31. World Health Organization. *Rabies Fact Sheet*. Geneva: World Health Organization. [No. 99, updated 2015 Sep] Available from: http://www.who.int/mediacentre/factsheets/fs099/en/
32. Al-Ani FK, El-Qaderi S, Hailat NQ, Razziq R, Al-Darraji AM. Human and animal brucellosis in Jordan between 1996 and 1998: a study. *Rev Sci Tech* (2004) **23**(3):831–40.
33. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. *Lancet Infect Dis* (2006) **6**(2):91–9. doi:10.1016/S1473-3099(06)70382-6
34. Jordan Ministry of Agriculture. *Annual Reports 1989-2000*. Amman: Department of Animal Production and Health, Ministry of Agriculture (2000).
35. Musallam II, Abo-Shehada M, Omar M, Guitian J. Cross-sectional study of brucellosis in Jordan: prevalence, risk factors and spatial distribution in small ruminants and cattle. *Prev Vet Med* (2015) **118**(4):387–96. doi:10.1016/j.prevetmed.2014.12.020
36. The Eastern Mediterranean Public Health Network. *EMPHNET Newsletter*. (2015). Issue 19. Available from: http://emphnet.net/wp-content/uploads/2015/08/EMPHNET_Newsletter_19.pdf

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 24 July 2015
doi: 10.3389/fmicb.2015.00765

Molecular evidence of a *Trypanosoma brucei gambiense* sylvatic cycle in the human african trypanosomiasis foci of Equatorial Guinea

*Carlos Cordon-Obras[1], Yasmin Fermin Rodriguez[2], Amalia Fernandez-Martinez[2], Jorge Cano[3], Nicolas Ndong-Mabale[4], Policarpo Ncogo-Ada[4], Pedro Ndongo-Asumu[4], Pilar Aparicio[2], Miguel Navarro[1], Agustin Benito[2] and Jean-Mathieu Bart[1,2]**

[1] Consejo Superior de Investigaciones Científicas, Instituto de Parasitologia y Biomedicina Lopez Neyra, Granada, Spain, [2] Centro Nacional de Medicina Tropical, Instituto de Salud Carlos III, Madrid, Spain, [3] Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK, [4] Centro de Referencia para el Control de Endemias, Instituto de Salud Carlos III, Malabo, Equatorial Guinea

OPEN ACCESS

Edited by:
Nathan Wolfe,
Metabiota, USA

Reviewed by:
Li Xu,
Cornell University, USA
Malgorzata Anna Mikaszewska-Sokolewicz,
Medical University of Warsaw, Poland

***Correspondence:**
Jean-Mathieu Bart,
Centro Nacional de Medicina Tropical, Instituto de Salud Carlos III, Calle Sinesio Delgado 5, 28029 Madrid, Spain
jmbart@isciii.es

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: 20 May 2015
Accepted: 13 July 2015
Published: 24 July 2015

Citation:
Cordon-Obras C, Rodriguez YF, Fernandez-Martinez A, Cano J, Ndong-Mabale N, Ncogo-Ada P, Ndongo-Asumu P, Aparicio P, Navarro M, Benito A and Bart J-M (2015) Molecular evidence of a Trypanosoma brucei gambiense sylvatic cycle in the human african trypanosomiasis foci of Equatorial Guinea.
Front. Microbiol. 6:765.
doi: 10.3389/fmicb.2015.00765

Gambiense trypanosomiasis is considered an anthroponotic disease. Consequently, control programs are generally aimed at stopping transmission of *Trypanosoma brucei gambiense* (*T. b. gambiense*) by detecting and treating human cases. However, the persistence of numerous foci despite efforts to eliminate this disease questions this strategy as unique tool to pursue the eradication. The role of animals as a reservoir of *T. b. gambiense* is still controversial, but could partly explain maintenance of the infection at hypo-endemic levels. In the present study, we evaluated the presence of *T. b. gambiense* in wild animals in Equatorial Guinea. The infection rate ranged from 0.8% in the insular focus of Luba to more than 12% in Mbini, a focus with a constant trickle of human cases. The parasite was detected in a wide range of animal species including four species never described previously as putative reservoirs. Our study comes to reinforce the hypothesis that animals may play a role in the persistence of *T. b. gambiense* transmission, being particularly relevant in low transmission settings. Under these conditions the integration of sustained vector control and medical interventions should be considered to achieve the elimination of gambiense trypanosomiasis.

Keywords: *Trypanosoma brucei gambiense*, wild fauna, reservoir, human African trypanosomiasis, sleeping sickness, Equatorial Guinea

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a tropical disease caused by two subspecies of the protozoan flagellate, *Trypanosoma brucei* s.l.: *T. b. gambiense,* and *T. b. rhodesiense*. Both are closely related on a genetic level, but have important phenotypic differences regarding their epidemiology, transmission, clinical features, and distribution. The vector for *T. brucei* s.l. and other African trypanosomes is the tsetse fly (order Diptera, genus *Glossina*), but each subgenus of *Glossina* sp. has different susceptibility to diverse trypanosome species (Ashford, 2003; Kuzoe and Schofield, 2004), conditioning the distribution of both human and animal trypanosomiasis (World Health Organization, 2013).

Trypanosoma brucei gambiense is responsible for more than 97% of HAT cases and is spread over Central and West Africa (Simarro et al., 2012). In contrast with *T. b. rhodesiense*, which is known to circulate in domestic and wild fauna (Franco et al., 2014a), *T. b. gambiense* has traditionally been considered an anthroponosis, i.e., human beings are the main reservoir of the parasite (World Health Organization, 2013). Consistent with this assumption, control interventions based on detection and treatment of human cases have proven to be sufficient to drastically reduce the prevalence, even in the absence of vector control (Simarro et al., 2006; Franco et al., 2014b). Despite the effectiveness of these actions, virulent outbreaks have occurred over the past century after abandoning these control measures (Steverding, 2008). Thus, complete elimination was only rarely achieved in particular foci. The persistence of infection, even when no human cases were reported for years, has been attributed to different causes such as asymptomatic parasite carriers (Jamonneau et al., 2012), continuous reintroduction due to movements of both human and vector populations (Courtin et al., 2008), and inherent limitations of the surveillance system (Louis et al., 2008). An increasing number of studies have shown that domestic and wild fauna could also harbor *T. b. gambiense* even though it still remains unclear whether animals can host the parasite long enough to play an important role in the transmission of Gambiense HAT (Gibson et al., 1978; Noireau et al., 1986; Herder et al., 2002; Njiokou et al., 2006; Simo et al., 2006; Cordon-Obras et al., 2009).

Equatorial Guinea, a small country located in the Guinea Gulf, has four historical HAT foci; three in the coastal region of the continental part (Kogo, Mbini, and Campo) and one in Bioko Island (Luba) that is currently considered free of HAT (Simarro et al., 2006). Since the mid-1980s, the number of HAT cases diagnosed is very low thanks to the implementation of control campaigns based on systematic screening of endemic populations and treatment of patients. In spite of the considerable and sustained efforts, a continuous trickle of cases is reported every year in the mainland foci, Kogo, and Mbini (Franco et al., 2014a). In a previous study, we suggested that the epidemiological situation in both foci might follow different patterns. Whereas peri-domestic fauna could act as a reservoir of infection in Mbini (DNA of *T. b. gambiense* were detected in seven animals), we did not find *T. b. gambiense* in livestock from the Kogo focus (Cordon-Obras et al., 2009). In Luba, where no human cases have been reported since 1995 (Simarro et al., 2006), we were unable to detect the parasite in domestic fauna, although we found a positive tsetse fly sample, demonstrating that *T. b. gambiense* is still present in Bioko Island (Cordon-Obras et al., 2010). The persistence of infection in Kogo and Luba foci could not be explained by our data from domestic animal samples. Therefore, we hypothesized the existence of alternative epidemiological cycles. In such ecological context, where the forest represents more than 78.5% (22,000 km^2) of the total land surface of the country, wild animals would represent potential reservoir candidates.

In the present work, employing the same molecular tools previously used (Cordon-Obras et al., 2009, 2010), we performed a molecular screening of *T. b. gambiense* in wild fauna and considered its possible role in the maintenance of the parasite in the historical foci of Equatorial Guinea. We established potential scenarios depending on the specificity of each focus, highlighting their differences, and discussed the epidemiological implications of the occurrence of a sylvatic transmission cycle for *T. b. gambiense*.

Materials and Methods

Study Area

Four HAT foci from Equatorial Guinea were studied: Luba (at the southwest of Bioko Island), Rio Campo (north of the mainland region, bordering with Cameroon), Mbini (central mainland coast), and Kogo (southern mainland coast, bordering with Gabon), (**Figure 1**). Bioko Island is located more than 200 km from the mainland. Rainforest and abandoned cocoa plantations are widespread in Luba district (Simarro et al., 1990), covering a surface of 700 km^2. Continental foci are located across the coastal region and have a typical maritime equatorial climate of four seasons. Campo extends along the Ntem River, which delineates the border between Cameroon and Equatorial Guinea, and is mostly covered by the equatorial rainforest (Simo et al., 2014). Mbini is located close to the mouth of the Wele River, whereas Kogo limits with Gabon separated by a natural boundary created by the Muni estuary. Mangrove swamp and rainforest are the predominant ecosystems in the Kogo and Mbini foci.

Sampling Strategy

From June 2012 to March 2014, surveys were conducted to collect blood samples from wild fauna. Samples were obtained either in local markets (**Figure 2A**) or directly from hunters and trappers (**Figure 2B**). Our team had absolutely no control over the species selected by hunters. The blood taken by venepuncture (**Figure 2C**) was spread on Whatman paper, left to dry in darkness and then stored at 4°C until processing in the reference laboratory at the National Center of Tropical Medicine, Madrid (Institute of Health Carlos III). Each filter paper was labeled to link the blood sample with the additional information recorded: hunting area, date of capture, data, and place of blood sampling (**Figure 2D**). Each sample was stored separately to avoid cross-contamination. Ethical approval was obtained by the Ministry of Health and Social Welfare and Veterinary Service from the continental region (Ministry of Agriculture, Forestry and Environment). The study adhered to the institutions' guidelines for animal hunter game.

Host Species Identification

Wild animals captured by hunters and trappers and brought to the village markets were identified using a systematic key (Dorst and Dandelot, 1997). Molecular confirmation was performed by cytochrome b sequencing according to the conditions previously described (Steuber et al., 2005) when the species identification was problematic. This validation was also performed on *T. b. gambiense*-positive samples in order to accurately define the possible reservoir hosts.

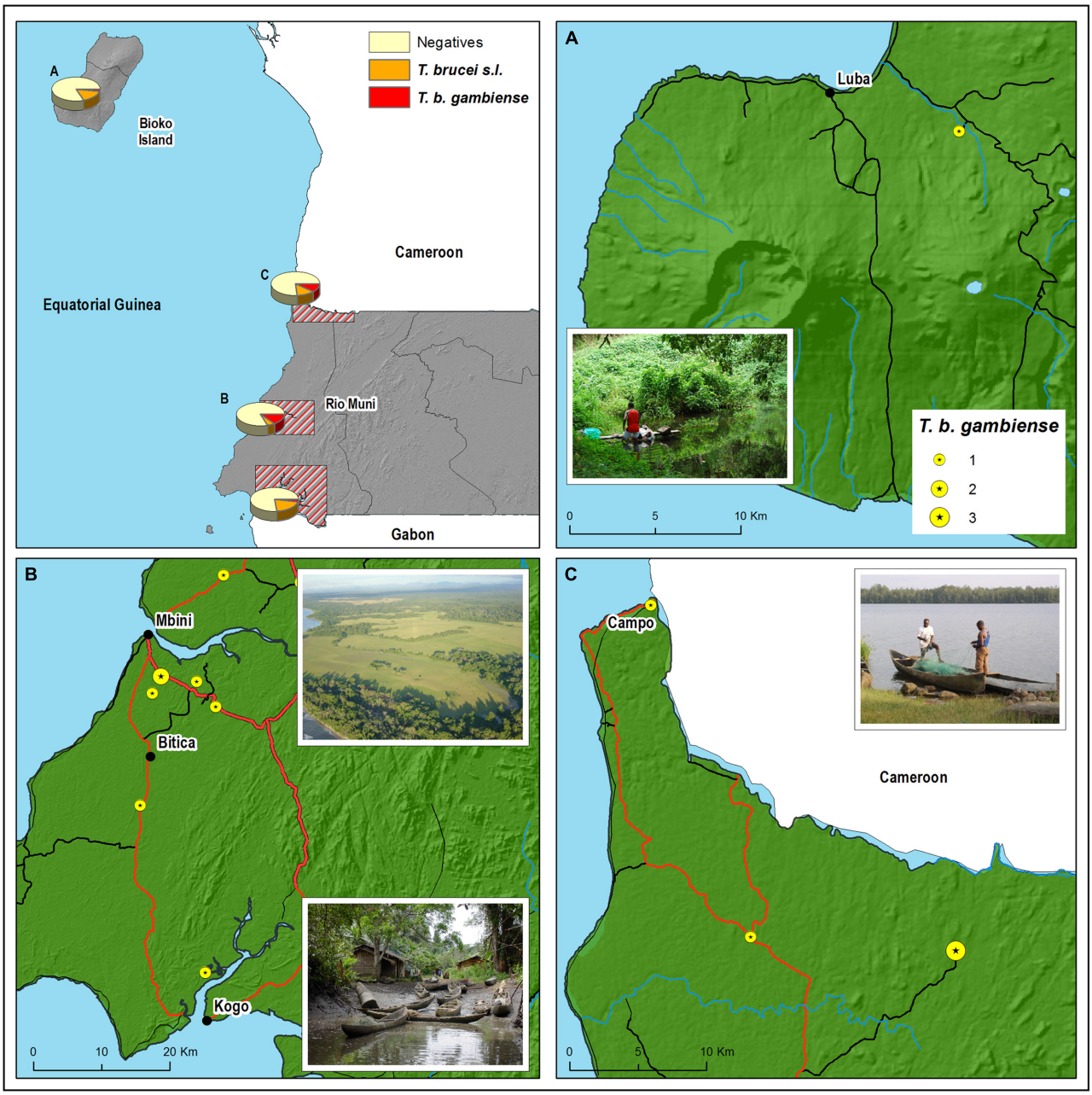

FIGURE 1 | Geographical distribution of the *Trypanosoma brucei gambiense (T. b. gambiense)*-positive animals according to the place where they were killed or trapped in (A) Luba, (B) Kogo and Mbini, and (C) Rio Campo. Main and secondary roads are represented in red and black lines, respectively.

Parasite Molecular Diagnosis

DNA was extracted from the samples collected employing the SpeedTools DNA Extraction kit (Biotools, Madrid, Spain) according to the manufacturer's instructions, DNA template (1 μl) was submitted to species-specific PCR for *T. brucei* s.l. using the oligonucleotides described by Moser et al. (1989) that target the palindromic 177 bp satellite of mini-chromosomes. Positive samples for 177 bp PCR were submitted to a *T. b. gambiense* group 1-specific nested-PCR using the primers described elsewhere (Cordon-Obras et al., 2009) which amplify the known specific TgsGP gene (Berberof et al., 2001) considered to be the gold standard method for the identification of this subspecies. For both PCRs, the conditions were as follows: 1x PCR Reaction Buffer [7.5 mM Tris-HCl -pH 9-, 1.5 mM $MgCl_2$, 5 mM KCl, and 2 mM $(NH_4)_2SO_4$], 200 μM of each deoxynucleotide (dNTP), primers at 1 μM, and 1 U of Certamp Complex Enzyme Mix (Biotools, Madrid, Spain) in a final volume of 25 μl. The amplification programs were set as follows: for *T. brucei* s.l.,

FIGURE 2 | Sampling strategy. Blood samples from wild animals were collected from market **(A)**, or directly from the hunters **(B)**. The blood taken by venepuncture **(C)** was then spread on Whatman paper. **(D)** Filter paper with blood sample was labeled and stored separately to avoid cross-contamination.

3 min at 95°C for initial DNA denaturation, 35 cycles of 95°C (30 s), 60°C (30 s), and 72°C (1 min), and a final extension step at 72°C (5 min). For the first reaction of *T. b. gambiense,* the fixed program was an initial denaturation step at 95°C (5 min), 35 cycles at 94°C (30 s), 55°C (1 min), and 72°C (3 min), with a final extension step at 72°C (5 min). The program for the nested reaction was identical. Negative (double distilled water) and positive [genomic DNA of *T. b. gambiense* ELIANE strain - MHOM/CI/52/ITMAP 2188- (Turner et al., 2004)] controls were systematically added to each assay. The amplification products were separated by electrophoresis in a 2% agarose gel stained with 1x RedSafe^{TM} Nucleic Acid Staining Solution (Intron Biotechnology, Inc.) and photographed under UV light. All positive samples for *T. b. gambiense* and a number of negative samples randomly selected were submitted to a second round of diagnosis performed blindly by a different researcher. All samples yielded a second positive result, confirming the robustness and reproducibility of our diagnostic method. Rigorous measures were employed to prevent contamination of the PCR reaction by establishment of a unidirectional physical workflow (pre-PCR–post-PCR).

Statistical Analysis

Prevalence of *T. brucei* s.l. and *T. b. gambiense* infections by focus were compared through Pearson's Chi-square test. Data analysis was conducted by using SPSS software (version 16.0.1, SPSS Inc., Chicago, IL, USA).

Results

Sample Collection

A total of 288 samples were collected within the four foci (121 in Luba, 66 in Mbini, 45 in Kogo, and 56 in Rio Campo) from June 2012 to March 2014. The complete animal records are provided as supplemental information (Supplementary Table S1). They included 26 animal species, 98.3% belonging to mammals, since they are the most frequent targets of hunting activities. Among mammals, the most represented orders were *Rodentia* and *Artiodactyla* (128 and 109 samples, respectively) given that the preferred preys in these foci are the African brush-tailed porcupine (*Atherurus africanus*), the African giant pouched rat (*Cricetomys gambianus*), and the hoofed blue duiker (*Cephalophus monticola*). *Primates* (29), *Carnivora* (13), and *Pholidota* (4) completed the mammal set. Four samples from reptiles and one from a bird was also analyzed.

Trypanosoma brucei Infection Rate

Trypanosoma brucei s.l. infection in the wild fauna was ubiquitous and homogenous in the four historical HAT foci of Equatorial Guinea, with infection rates ranging from 16.5% in Luba to 24.4% in Kogo, with 19.7% in Mbini and 23.2% in Rio Campo (**Table 1**). *Rodentia* (25) and *Artiodactylia* (19) represented 77.2% of the infected animals (**Table 2**). No statistical association was found between *T. brucei* prevalence and focus ($X^2 = 1.84$, p-value $= 0.61$).

A total of 15 samples were positive for the TgsGP nested-PCR. To ensure the specificity of these bands, the PCR products were sequenced. They all presented 100% homology with the TgsGP gene (DAL 972 reference strain). *T. b. gambiense* was detected in all foci, but with a heterogeneous pattern. Infection rate of *T. b. gambiense* was 12.1 and 8.8%, in Mbini, and Rio Campo, respectively, and significantly lower in Luba (0.8%) and Kogo (2.2%; $X^2 = 13.48$, p-value <0.01). No statistical association was found between prevalence of *T. brucei* or *T. b. gambiense* and

TABLE 1 | Distribution of *Trypanosoma brucei* s.l. and *Trypanosoma brucei gambiense* (*T. b. gambiense*) among wild fauna in different foci of Equatorial Guinea.

Focus	*T. brucei* s.l. no. (%)	*T. b. gambiense* no. (%)
Luba	20/121 (16.5)	1/121 (0.8)
Mbini	13/66 (19.7)	8/66 (12.1)
Kogo	11/45 (24.4)	1/45 (2.2)
Rio Campo	13/56 (23.2)	5/56 (8.9)
Total	**57/288 (19.8)**	**15/288 (5.2)**

TABLE 2 | Distribution of *T. brucei* s.l. and *T. b. gambiense* according to animal species.

Focus	Species		Intra-species Prevalence (%)	
	Common name	Scientific name	*T. brucei s.l.*	*T. b. gambiense*
Luba	***Carnivora***			
	Small-spotted genet	*Genetta servalina*	1/2 (50)	–
	Rodentia			
	Red-legged sun squirrel	*Heliosciurus rufobrachium**	3/16 (18.8)	1/16 (6.3)
	Brush-tailed porcupine	*Atherurus africanus*	5/34 (14.7)	–
	African giant pouched rat	*Cricetomys gambianus*	3/25 (12)	–
	Artiodactyla			
	Blue Duiker	*Cephalophus monticola*	7/35 (20)	–
	Reptiles			
	African Rock Python	*Python sebae*	1/1 (100)	–
Mbini	***Primates***			
	Red-tailed monkey	*Cercopithecus ascanius**	1/2 (50)	1/2 (50)
	Grey-cheeked mangabey	*Lophocebus albigena**	1/1 (100)	1/1 (100)
	Greater spot-nosed monkey	*Cercopithecus nictitans*	1/5 (20)	–
	Carnivora			
	Small-spotted genet	*Genetta servalina*	1/1 (100)	1/1 (100)
	African palm civet	*Nandinia binotata*	2/3 (66.7)	1/3 (33.3)
	Rodentia			
	Brush-tailed porcupine	*Atherurus africanus*	3/14 (21.4)	3/14 (21.4)
	African giant pouched rat	*Cricetomys gambianus*	1/6 (16.7)	1/6 (16.7)
	Artiodactyla			
	Blue Duiker	*Cephalophus monticola*	3/19 (15.8)	–
Kogo	***Carnivora***			
	Small-spotted genet	*Genetta servalina*	1/1 (100)	–
	Rodentia			
	Brush-tailed porcupine	*Atherurus africanus*	4/5 (80)	–
	Artiodactyla			
	Blue Duiker	*Cephalophus monticola*	5/22 (22.7)	1/22 (4.5)
	Red River Hog	*Potamochoerus porcus*	1/1 (100)	–
Rio Campo	***Primates***			
	Greater spot-nosed monkey	*Cercopithecus nictitans*	3/4 (75)	1 /4 (25)
	Carnivora			
	African Lisang	*Poiana richardsonii**	1/2 (50)	1/2 (50)
	Rodentia			
	Brush-tailed porcupine	*Atherurus africanus*	4/11 (36.4)	1/11 (9.1)
	African giant pouched rat	*Cricetomys gambianus*	2/7 (28.6)	–
	Artiodactyla			
	Blue Duiker	*Cephalophus monticola*	3/9 (33.3)	2/9 (22.2)

**Species detected as putative T. b. gambiense reservoir for the first time in this study.*

the host order ($X^2 = 7.91$, p-value $= 0.34$ and $X^2 = 11.85$, p-value $= 0.1$, for *T. brucei* and *T. b. gambiense*, respectively).

Overall, 10 mammals species were found to harbor *T. b. gambiense*, belonging to four orders (*Rodentia*, *Artiodactyla*, *Carnivora,* and *Primates*; **Table 2**). Similarly to *T. brucei* s.l. infection, 60% of the *T. b. gambiense* cases were distributed among rodents and artiodactyls; carnivores and primates represented the remaining 40%. Among those positives for *T. b. gambiense*, we identified four species so far never reported as putative reservoirs of this parasite; these were *Heliosciurus rufobrachium* (Red-legged sun squirrel), *Cercopithecus ascanius* (red-tailed monkey), *Lophocebus albigena* (gray-cheeked mangabey), and *Poiana richardsonii* (African lisang).

Discussion

The four historical foci of Equatorial Guinea depict the diversity of epidemiological settings. In the past, Luba was the most important focus of HAT in this country, reporting hundreds

of cases per year (Simarro et al., 1990). The implementation of control activities based on active detection and systematic treatment of patients in 1985 drove to the elimination of the human disease one decade later (Simarro et al., 2006). Kogo and Mbini always had a marginal importance compared to Luba in terms of HAT incidence. However, despite similar control measures were implemented the complete elimination has not been yet achieved in the mainland foci. On the other hand, Campo has historically reported few cases (10 cases since 2000 according to Equatorial Guinea Ministry of Health) even in the absence of control interventions in the Guinean part of the focus (Simo et al., 2014). One might speculate that the isolation of Bioko Island would have contributed to the success of control interventions, whereas factors such as population movements, reintroduction of infected vectors, and insufficient coverage in the campaigns of active screening might be leading to a steady occurrence of cases in the mainland foci.

In this study, we demonstrate the presence of *T. b. gambiense* in different species of wild fauna in all the four endemic HAT foci of Equatorial Guinea. These findings support our previous works, where we concluded that diverse eco-epidemiological scenarios, involving different animal reservoirs, might underlie the variety of transmission patterns for *T. b. gambiense* in Equatorial Guinea.

We already reported that *T. b. gambiense* was present in domestic livestock from Mbini but not Kogo (Cordon-Obras et al., 2009). According to these results, we speculated on the occurrence of a peri-domestic transmission cycle in Mbini, involving livestock often circulating close to populations. In the present study, while the *T. brucei* s.l. infection rate is similar in all foci, we observed a significantly higher prevalence of *T. b. gambiense* in wild fauna of Mbini compared to Kogo. These data suggest an additional transmission activity in a sylvatic cycle along with the peri-domestic cycle previously hypothesized (Cordon-Obras et al., 2009). It is noteworthy that the prevalence of *T. b. gambiense* infection in wild fauna in Mbini focus was higher than previously reported in peri-domestic livestock (12.1% vs. 2%; Cordon-Obras et al., 2009), which could be explained by a greater preference of vector population for wild fauna or simply a greater availability of wildlife versus livestock. Further studies aiming to describe the feeding preferences of tsetse flies will be conducted to clear up this question. In Kogo, where new HAT cases are actually rare, *T. b. gambiense* has been detected in only one wild animal out of 45 screened, indicating that (i) a sylvatic cycle can occur in this focus, but (ii) parasite transmission level seems low.

Campo focus, shared by Equatorial Guinea and Cameroon, has particular epidemiological features. Although this focus has remained active in both sides of the border, only a handful of HAT cases have been reported over the last years in the Guinean part. Subsequently, unlike the other foci, active screening has not been undertaken in a regular basis in Campo. Some studies have reported the presence of *T. b. gambiense* in animals and the occurrence of human infections in the Cameroonian side of the border, even though at low level (Morlais et al., 1998; Penchenier et al., 1999; Herder et al., 2002). Our data reveal 8.9% of infection rate in wild fauna, above the prevalence reported in wild (0.6%; Njiokou et al., 2006) and domestic (4.4%; Njiokou et al., 2010) animals on the Cameroonian side. The different molecular markers used in the screening or the diverse epidemiological patterns can in part explain these contrasted results. It is noteworthy that in the Cameroonian focus of Fontem, it was described a high *T. b. gambiense* prevalence in pigs (14.8%; Simo et al., 2006), suggesting different epidemiological scenarios between foci from Cameroon as we noticed in Equatorial Guinea. According to our findings we might conclude that *T. b. gambiense* is mainly circulating in a sylvatic cycle in Campo, and the absence of human cases is likely due to the low population density in the area. However, a plan to communicate both sides of Ntem river by road (Economic Community of Central African States source, see http://www.ceeac-eccas.org) and the development of Campo municipality in the Guinean part would undoubtedly result in an increase of human presence in the area and in turn, a greater risk of exposure to *T. b. gambiense* infection.

The Luba focus has not reported autochthonous HAT cases since 1995 (Simarro et al., 2006). However, in a recent publication we demonstrated the persistence of *T. b. gambiense* in tsetse flies (Cordon-Obras et al., 2010). Given that no vertical transmission of the parasite has been reported in the tsetse fly and the apparent lack of infection in the local livestock, we suggested that a wild cycle could be the responsible for the maintenance of *T. b. gambiense*, even after the absence of HAT cases for decades (Simarro et al., 2006). In the present work, we found a positive sample of *T. b. gambiense* amongst the blood samples gathered from wild animals (0.8% prevalence). The epidemiological scenario of Luba seems to be quite similar to Kogo focus, where HAT cases are now rarely reported, *T. b. gambiense* is apparently absent in domestic fauna and this parasite is found at low prevalence in wild fauna. All these data together strongly suggest the existence of a sylvatic cycle with low activity in both foci which may hinder a definitive eradication of the disease.

Our data also revealed homogenous prevalence of *T. brucei* s.l. in all the studied foci, but this pattern is not observed in *T. b. gambiense*. This could be explained by the fact that control measures taken over human reservoirs and the HAT epidemiological features of each focus affect *T. b. gambiense*, but not *T. b. brucei* which is, besides, much more easily acquired and transmitted by tsetse flies (Kuzoe and Schofield, 2004).

As sampling is biased due to the methods, the species that host the parasite are similar to those found in other African foci (Njiokou et al., 2006), i.e., those that are targets of hunting and trapping. In spite of this bias, we identified some species that have never been previously reported as reservoirs of *T. b. gambiense*. In addition, we cannot rule out that more animal species may harbor *T. b. gambiense*. Indeed, there is no apparent association between the rate of infection and taxonomic order in either *T. b. brucei* or *T. b. gambiense*, suggesting that these parasites can adapt to a wide range of species with no evident preference. This is consistent with previous findings that point out the ability of trypanosomes to proliferate in several species in both *in vivo* and *in vitro* experiments (Bitter et al., 1998; Herder et al., 2002; Njiokou et al., 2006; Cordon-Obras et al., 2013). Besides, it is well known that most of the other human pathogenic kinetoplastids like *T. b. rhodesiense*, *T. cruzi,* or *Leishmania* sp. are able to

circulate in a wide range of animal species (Sobrino et al., 2008; Oda et al., 2014; Zanet et al., 2014).

Despite the provided evidences, we assume that detecting *T. b. gambiense* in wild fauna does not definitively prove that these animals represent relevant reservoirs for the maintenance of the parasites that are then able to infect humans. Nevertheless, epidemiological arguments such as the higher significant prevalence in Mbini (the most active HAT focus) and Campo (where no control measures were implemented), the previously detected presence of the parasite in tsetse flies in Luba (but not in humans or domestic fauna; Cordon-Obras et al., 2010), and the fact that most of the animals had already been detected previously as being positive for *T. b. gambiense* (Njiokou et al., 2006), support the existence of sylvatic cycles. If that is correct, it would remain to be elucidated whether both animal and human cycles are linked. To answer this question, we will further analyze by microsatellite approach the samples used in this study and others from HAT patients concurrently isolated from the same foci. The accurate genetic identification of the same populations circulating in both cycles would provide a strong argument in support of our hypothesis.

The lack of an appropriate description of possible reservoirs of *T. b. gambiense* is a fundamental drawback in the design of control strategies. Wild fauna is a link in the epidemiological chain that is much more difficult to control, and interventions directed against vector would probably be the way to interrupt parasite transmission. Animal reservoirs often mask the infection of a parasitic or viral disease between outbreak periods. Over the 20th century, HAT progression has followed a similar pattern (Steverding, 2008), with alternating periods of low incidence and virulent outbreaks. The intensive control efforts focused on human patients have been always very effective to control the disease, but rarely sufficient to definitively eliminate it in any focus. Recently, based on mathematical models of gambiense HAT transmission involving humans, peri-domestic and wild animals in the Cameroonian focus of Bipindi, a study suggested the crucial role of animals in the maintenance of *T. b. gambiense* (Funk et al., 2013). In Equatorial Guinea, if alternative reservoirs are ruled out and neglected in control programs, *T. b. gambiense* could be maintained in the wild cycle as has probably occurred in Luba, and may lead to virulent outbreaks in the future as has happened in the past (Steverding, 2008). As previously addressed by other authors (Gouteux and Artzrouni, 1996; Solano et al., 2013), vector control will be essential to a complete elimination and we strongly recommend its introduction in eradication programs to complement the active screening focused in the human patients.

Acknowledgments

We thank the hunter teams of Equatorial Guinea and the staff of the National Sleeping Sickness Control Programme. We wanted to extend our thanks to the authorities of the Ministry of Health and Social Welfare of Equatorial Guinea and the Spanish Agency for International Development Cooperation (AECID). We are grateful to Dr. Vincent Jamonneau (IRD) for providing valuable comments of the manuscript. J-MB is supported by a Miguel Servet Fellowship (CP09/00300) and funded by "Fondo de Investigación Sanitaria"(FIS) PI10/01128. AB and MN are granted by ISCIII -Subdirección General de Redes y Centros de InvestigaciónCooperativa (RICET) RD12/0018/0001 and RD12/0018/0015, respectively.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00765

References

Ashford, R. W. (2003). When is a reservoir not a reservoir? *Emerg. Infect. Dis.* 9, 1495–1496. doi: 10.3201/eid0911.030088

Berberof, M., Perez-Morga, D., and Pays, E. (2001). A receptor-like flagellar pocket glycoprotein specific to *Trypanosoma brucei gambiense*. *Mol. Biochem. Parasitol.* 113, 127–138. doi: 10.1016/S0166-6851(01)00208-0

Bitter, W., Gerrits, H., Kieft, R., and Borst, P. (1998). The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. *Nature* 391, 499–502. doi: 10.1038/35166

Cordon-Obras, C., Berzosa, P., Ndong-Mabale, N., Bobuakasi, L., Buatiche, J. N., Ndongo-Asumu, P., et al. (2009). *Trypanosoma brucei gambiense* in domestic livestock of Kogo and Mbini foci (Equatorial Guinea). *Trop. Med. Int. Health* 14, 535–541. doi: 10.1111/j.1365-3156.2009.02271.x

Cordon-Obras, C., Cano, J., Gonzalez-Pacanowska, D., Benito, A., Navarro, M., and Bart, J. M. (2013). *Trypanosoma brucei gambiense* adaptation to different mammalian sera is associated with VSG expression site plasticity. *PLoS ONE* 8:e85072. doi: 10.1371/journal.pone.0085072

Cordon-Obras, C., Garcia-Estebanez, C., Ndong-Mabale, N., Abaga, S., Ndongo-Asumu, P., Benito, A., et al. (2010). Screening of *Trypanosoma brucei gambiense* in domestic livestock and tsetse flies from an insular endemic focus (Luba, Equatorial Guinea). *PLoS Negl. Trop. Dis.* 4:e704. doi: 10.1371/journal.pntd.0000704

Courtin, F., Jamonneau, V., Duvallet, G., Garcia, A., Coulibaly, B., Doumenge, J. P., et al. (2008). Sleeping sickness in West Africa (1906-2006): changes in spatial repartition and lessons from the past. *Trop. Med. Int. Health* 13, 334–344. doi: 10.1111/j.1365-3156.2008.02007.x

Dorst, J., and Dandelot, P. (1997). *Guide Des Mammifères d'Afrique*, 2nd Edn. Neuchatel: Delachaux & Niesflé.

Franco, J. R., Simarro, P. P., Diarra, A., and Jannin, J. G. (2014a). Epidemiology of human African trypanosomiasis. *Clin. Epidemiol.* 6, 257–275. doi: 10.2147/CLEP.S39728

Franco, J. R., Simarro, P. P., Diarra, A., Ruiz-Postigo, J. A., and Jannin, J. G. (2014b). The journey towards elimination of gambiense human African trypanosomiasis: not far, nor easy. *Parasitology* 141, 748–760. doi: 10.1017/S0031182013002102

Funk, S., Nishiura, H., Heesterbeek, H., Edmunds, W. J., and Checchi, F. (2013). Identifying transmission cycles at the human-animal interface: the role of animal reservoirs in maintaining gambiense human african trypanosomiasis. *PLoS Comput. Biol.* 9:e1002855. doi: 10.1371/journal.pcbi.1002855

Gibson, W., Mehlitz, D., Lanham, S. M., and Godfrey, D. G. (1978). The identification of *Trypanosoma brucei gambiense* in Liberian pigs and dogs by isoenzymes and by resistance to human plasma. *Tropenmed. Parasitol.* 29, 335–345.

Gouteux, P. J., and Artzrouni, M. (1996). [Is vector control needed in the fight against sleeping sickness? A biomathematical approach]. *Bull. Soc. Pathol. Exot.* 89, 299–305.

Herder, S., Simo, G., Nkinin, S., and Njiokou, F. (2002). Identification of trypanosomes in wild animals from southern Cameroon using the polymerase chain reaction (PCR). *Parasite* 9, 345–349. doi: 10.1051/parasite/2002094345

Jamonneau, V., Ilboudo, H., Kabore, J., Kaba, D., Koffi, M., Solano, P., et al. (2012). Untreated human infections by *Trypanosoma brucei gambiense* are not 100% fatal. *PLoS Negl. Trop. Dis.* 6:e1691. doi: 10.1371/journal.pntd.0001691

Kuzoe, F. A. S., and Schofield, C. J. (2004). *Strategic Review of Traps and Targets for Tsetse and African Trypanosomiasis Control*. Report of Special Programme for Research and Training in Tropical Diseases TDR/IDE/TRY/05.1. Geneva: World Health Organization, 58.

Louis, F. J., Kohagne Tongue, L., Ebo, O. E. V., and Simarro, P. P. (2008). [Organizing an active screening campaign for human African trypanosomiasis due to *Trypanosoma brucei gambiense*]. *Med. Trop. (Mars)* 68, 11–16.

Morlais, I., Grebaut, P., Bodo, J. M., Djoha, S., Cuny, G., and Herder, S. (1998). Detection and identification of trypanosomes by polymerase chain reaction in wild tsetse flies in Cameroon. *Acta Trop.* 70, 109–117. doi: 10.1016/S0001-706X(98)00014-X

Moser, D. R., Cook, G. A., Ochs, D. E., Bailey, C. P., Mckane, M. R., and Donelson, J. E. (1989). Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* 99 (Pt 1), 57–66. doi: 10.1017/S0031182000061023

Njiokou, F., Laveissiere, C., Simo, G., Nkinin, S., Grebaut, P., Cuny, G., et al. (2006). Wild fauna as a probable animal reservoir for *Trypanosoma brucei gambiense* in Cameroon. *Infect. Genet. Evol.* 6, 147–153. doi: 10.1016/j.meegid.2005.04.003

Njiokou, F., Nimpaye, H., Simo, G., Njitchouang, G. R., Asonganyi, T., Cuny, G., et al. (2010). Domestic animals as potential reservoir hosts of *Trypanosoma brucei gambiense* in sleeping sickness foci in Cameroon. *Parasite* 17, 61–66. doi: 10.1051/parasite/2010171061

Noireau, F., Gouteux, J. P., Toudic, A., Samba, F., and Frezil, J. L. (1986). [Epidemiological importance of the animal reservoir of *Trypanosoma brucei* gambiense in the Congo. 1. Prevalence of animal trypanosomiasis in the foci of sleeping sickness]. *Trop. Med. Parasitol.* 37, 393–398.

Oda, E., Solari, A., and Botto-Mahan, C. (2014). Effects of mammal host diversity and density on the infection level of Trypanosoma cruzi in sylvatic kissing bugs. *Med. Vet. Entomol.* 28, 384–390. doi: 10.1111/mve.12064

Penchenier, L., Grebaut, P., Eboo Eyenga, V., Bodo, J. M., Njiokou, F., Binzouli, J. J., et al. (1999). [The focus of human trypanosomiasis in Campo (Cameroon). History and endemic situation in 1998]. *Bull. Soc. Pathol. Exot.* 92, 185–190.

Simarro, P. P., Cecchi, G., Franco, J. R., Paone, M., Diarra, A., Ruiz-Postigo, J. A., et al. (2012). Estimating and mapping the population at risk of sleeping sickness. *PLoS Negl. Trop. Dis.* 6:e1859. doi: 10.1371/journal.pntd.0001859

Simarro, P. P., Franco, J. R., Ndongo, P., Nguema, E., Louis, F. J., and Jannin, J. (2006). The elimination of *Trypanosoma brucei gambiense* sleeping sickness in the focus of Luba, Bioko Island, Equatorial Guinea. *Trop. Med. Int. Health* 11, 636–646. doi: 10.1111/j.1365-3156.2006.01624.x

Simarro, P. P., Mas, J., Lancien, J., Ona Sima, F., Mateo, M. J., and Roche, J. (1990). [Epidemiology of human trypanosomiasis in the Luba focus, in Equatorial Guinea]. *Rev. Sanid. Hig. Publica (Madr)* 64, 517–534.

Simo, G., Asonganyi, T., Nkinin, S. W., Njiokou, F., and Herder, S. (2006). High prevalence of *Trypanosoma brucei gambiense* group 1 in pigs from the Fontem sleeping sickness focus in Cameroon. *Vet. Parasitol.* 139, 57–66. doi: 10.1016/j.vetpar.2006.02.026

Simo, G., Mbida Mbida, J. A., Ebo'o Eyenga, V., Asonganyi, T., Njiokou, F., and Grebaut, P. (2014). Challenges towards the elimination of Human African Trypanosomiasis in the sleeping sickness focus of Campo in southern Cameroon. *Parasit. Vectors* 7, 374. doi: 10.1186/1756-3305-7-374

Sobrino, R., Ferroglio, E., Oleaga, A., Romano, A., Millan, J., Revilla, M., et al. (2008). Characterization of widespread canine leishmaniasis among wild carnivores from Spain. *Vet. Parasitol.* 155, 198–203. doi: 10.1016/j.vetpar.2008.05.003

Solano, P., Torr, S. J., and Lehane, M. J. (2013). Is vector control needed to eliminate gambiense human African trypanosomiasis? *Front. Cell. Infect. Microbiol.* 3:33. doi: 10.3389/fcimb.2013.00033

Steuber, S., Abdel-Rady, A., and Clausen, P. H. (2005). PCR-RFLP analysis: a promising technique for host species identification of blood meals from tsetse flies (Diptera: Glossinidae). *Parasitol. Res.* 97, 247–254. doi: 10.1007/s00436-005-1410-y

Steverding, D. (2008). The history of African trypanosomiasis. *Parasit. Vectors* 1, 3. doi: 10.1186/1756-3305-1-3

Turner, C. M., Mclellan, S., Lindergard, L. A., Bisoni, L., Tait, A., and Macleod, A. (2004). Human infectivity trait in *Trypanosoma brucei*: stability, heritability and relationship to sra expression. *Parasitology* 129, 445–454. doi: 10.1017/S0031182004005906

World Health Organization. (2013). Control and surveillance of human African trypanosomiasis. *World Health Organ. Tech. Rep. Ser.* 1–237.

Zanet, S., Sposimo, P., Trisciuoglio, A., Giannini, F., Strumia, F., and Ferroglio, E. (2014). Epidemiology of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in *Rattus rattus* in absence of domestic reservoir and definitive hosts. *Vet. Parasitol.* 199, 247–249. doi: 10.1016/j.vetpar.2013.10.023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

ORIGINAL RESEARCH
published: 13 October 2015
doi: 10.3389/fpubh.2015.00232

Occurrence and diversity of clinically important *Vibrio* species in the aquatic environment of Georgia

*Tamar Kokashvili[1], Chris A. Whitehouse[2†], Ana Tskhvediani[1], Christopher J. Grim[3,4‡], Tinatin Elbakidze[1], Nino Mitaishvili[1‡], Nino Janelidze[1], Ekaterine Jaiani[1], Bradd J. Haley[4], Nino Lashkhi[1], Anwar Huq[4], Rita R. Colwell[3,4] and Marina Tediashvili[1]**

[1] G. Eliava Institute of Bacteriophages, Microbiology, and Virology, Tbilisi, Georgia, [2] United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, MD, USA, [3] Institute for Advanced Computer Studies, University of Maryland College Park, College Park, MD, USA, [4] Department of Cell Biology and Molecular Genetics, Maryland Pathogen Research Institute, University of Maryland College Park, College Park, MD, USA

Among the more than 70 different *Vibrio* species inhabiting marine, estuarine, and freshwater ecosystems, 12 are recognized as human pathogens. The warm subtropical climate of the Black Sea coastal area and inland regions of Georgia likely provides a favorable environment for various *Vibrio* species. From 2006 to 2009, the abundance, ecology, and diversity of clinically important *Vibrio* species were studied in different locations in Georgia and across seasons. Over a 33-month period, 1,595 presumptive *Vibrio* isolates were collected from the Black Sea (n = 657) and freshwater lakes around Tbilisi (n = 938). Screening of a subset of 440 concentrated and enriched water samples by PCR-electrospray ionization/mass spectrometry (PCR-ESI/MS) detected the presence of DNA from eight clinically important *Vibrio* species: *V. cholerae*, *V. parahaemolyticus, V. vulnificus, V. mimicus, V. alginolyticus, V. harveyi, V. metschnikovii*, and *V. cincinnatiensis.* Almost 90% of PCR/ESI-MS samples positive for *Vibrio species* were collected from June through November. Three important human-pathogenic *Vibrio* species (*V. cholerae, V. parahaemolyticus,* and *V. vulnificus*) were detected in 62.8, 37.8, and 21.4% of samples testing positive for *Vibrios*, respectively. The results of these activities suggest that natural reservoirs for human-pathogenic *Vibrios* exist in Georgian aquatic environments. Water temperature at all sampling sites was positively correlated with the abundance of clinically important *Vibrio* spp. (except *V. metschnikovii*), and salinity was correlated with species composition at particular Black Sea sites as well as inland reservoirs.

Keywords: aquatic environment, Black Sea, lakes, infection, *Vibrios*, conventional culture, direct detection, diversity

OPEN ACCESS

Edited by:
Nathan Wolfe, Metabiota, USA

Reviewed by:
Li Xu, Cornell University, USA
Carla Pruzzo, University of Genova, Italy

****Correspondence:***
Marina Tediashvili
m_tediash.ibmv@caucasus.net

[†]Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Army.

[‡]Present address:
Christopher J. Grim, U.S. Food and Drug Administration, Laurel, MD, USA; Nino Mitaishvili, Branch of Battelle Memorial Institute in Georgia, Tbilisi, Georgia

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *16 June 2015*
Accepted: *28 September 2015*
Published: *13 October 2015*

Citation:
Kokashvili T, Whitehouse CA, Tskhvediani A, Grim CJ, Elbakidze T, Mitaishvili N, Janelidze N, Jaiani E, Haley BJ, Lashkhi N, Huq A, Colwell RR and Tediashvili M (2015) Occurrence and diversity of clinically important Vibrio species in the aquatic environment of Georgia. Front. Public Health 3:232. doi: 10.3389/fpubh.2015.00232

INTRODUCTION

Vibrio species are ubiquitous and abundant in aquatic environments. They exist suspended in the water column, attached to plankton, and in the tissues or organs of various marine animals (1). The genus *Vibrio* includes more than 70 species, which are characterized as halophilic or non-halophilic according to their need for sodium chloride for growth (2, 3). At least 12 *Vibrio* species, including

V. cholerae, *V. parahaemolyticus*, and *V. vulnificus,* are pathogenic for humans and important to public health (4–7).

Among more than 200 known *V. cholerae* serotypes, serotypes O1 and O139 have epidemic and clinical importance. These serotypes are the causative agents of cholera, a severe diarrheal disease with 50–60% mortality in untreated cases. Cholera is contracted through consumption of contaminated food and water. Non-O1 and non-O139 *Vibrio* serotypes (also called non-agglutinable or NAG, serotypes) have also been implicated as etiologic agents of moderate to severe gastroenteritis in humans (2, 8, 9). *V. parahaemolyticus*, a halophilic *Vibrio* species, mainly causes gastroenteritis associated with consumption of contaminated seafood. In the last decade, epidemics of gastroenteritis in Southeast Asia, Japan, and North America have been linked to *V. parahaemolyticus* pandemic serotype O3:K6 (10, 11). Wound infections and septicemia caused by *V. vulnificus* carry a case fatality rate (CFR) as high as 50% in healthy patients; the CFR of *V. vulnificus* infections is even higher among immuno-compromised patients or those with liver disease (6). *V. metschnikovii* can cause gastroenteritis and wound infections leading to septicemia, while *V. fluvialis*, *Grimontia hollisae* (formerly *V. hollisae*), and *V. furnissii* typically cause gastroenteritis (12, 13). *V. alginolyticus* is commonly associated with ear infections, but can also cause respiratory infections and bacteremia (14, 15). *Photobacterium damselae* subsp. *damselae* (formerly *V. damsela*) causes opportunistic wound infections that can rapidly progress to a necrotizing condition, and ultimately to sepsis, which is fatal in 30–50% of human cases (16). A few cases of human illness (e.g., septicemia, meningitis, and diarrhea) have been associated with *V. cincinnatiensis* (17). *V. alginolyticus*, *P. damselae*, and *V. harveyi* are also opportunistic pathogens of economic significance in aquaculture, responsible for high mortality in cultured fish and shellfish, sometimes destroying an entire aquaculture operation (18).

Prevention and control of infections caused by *Vibrio* species pathogenic for humans depend on understanding their ecology, pathogenicity, and modes of transmission. A warm, subtropical climate – such as in the Black Sea coastal region of the South Caucasus – may support growth and multiplication of most *Vibrio* species (2, 19–21). A limited number of short reports are available on the abundance of NAG *Vibrio* species (more specifically, *V. parahaemolyticus* and *V. alginolyticus*) in some coastal areas of the Black Sea (9, 22–24). In addition, environmental surveillance studies have been performed to fill gaps in the understanding of the ecology of *V. cholerae* and other *Vibrio* species of public health importance in Georgia and Azerbaijan (25–29). However, the full range of disease-causing *Vibrio* species in aquatic environments in this region has never been comprehensively examined.

The Black Sea coastal zones of Georgia and water reservoirs around Tbilisi have traditionally been popular recreational zones; the number of international visitors to the country has drastically increased in the last decade. The combination of climate change (in particular, elevated air and surface water temperatures) and the increasing anthropogenic effects of tourism may increase the risk of emergence and spread of water-borne and food-borne infections (30). An increase in the frequency of enteric diseases with diarrhea has been registered in Georgia, especially in the Ajara region, which is near the Black Sea sampling sites used in this study (31); a significant portion of these infections with idiopathic etiology may have been caused by pathogenic *Vibrio* species.

In light of these factors, the aim of this study was to conduct environmental surveillance to assess the abundance and diversity of clinically important *Vibrio* species along the Georgian coastal zone of the Black Sea and in freshwater reservoirs near Tbilisi. The study also aimed to evaluate the efficacy of conventional methods used by Georgian public health laboratories to detect and identify pathogenic *Vibrios* in the aquatic environment. To our knowledge, this report presents the first detailed description of environmental parameters in relation to the prevalence and community structure of pathogenic *Vibrio* species in recreational waters in Georgia.

MATERIALS AND METHODS

Sample Sites and Collection

From June 2006 through October 2008, surface water samples were collected bi-weekly from July through September and monthly the rest of the year. Water samples were collected along the Georgian Black Sea coastal zone at four permanent stations located 50–100 m from the shore. Sample sites included two estuaries: Supsa (N 42°00.008′ E 41° 41.01′) and Chorokhi (N 41°36.116′ E 41° 34.021′); and two popular recreational/tourist attraction areas: Green Cape (N 41°41.91′ E 41° 42.01′) and Batumi Boulevard (N 41°39.570′ E 41°38.006′). Water samples were also collected 2–5 m from the shore of three inland reservoirs around Tbilisi: Kumisi Lake (Site 1: N 41°35.153′ E 044°51.591′; Site 2: N 41°34.839′ E 044°51.304′); Lisi Lake (Site 1: N 41°44.440′ E 044°44.261′; Site 2: N 41°44.483′ E 044°44.326′); and Tbilisi Sea (Site 1: N 41°46.150′ E 044°48.904′; Site 2: N 41°45.765′ E 044°50.308′). Sample locations are displayed in Figure S1 in Supplementary Material with coordinates listed in Table S1 in Supplementary Material.

Temperature, salinity, pH, conductivity, dissolved oxygen, and total dissolved solids were measured using a portable multi-log environmental meter (YSI Model 556 MPS, Yellow Springs, OH, USA), according to manufacturer's instructions. The physicochemical parameters of each sample were measured in triplicate, and the mean was recorded for laboratory analysis; seasonal temperature and salinity measurements for the sampling sites are given in Table S2 in Supplementary Material. At the time of sample collection at each site, 100 L of water was filtered through plankton nets (200, 64, and 10-μm mesh size) to collect concentrated plankton samples and plankton-free water (32, 33).

Bacterial Strains

Five *Vibrio* strains were obtained from the collection of the Institute Pasteur (CIP): *V. cholerae* CIP 62.13; *V. cholerae* CIP 55.91 (O1 classical); *V.* cholerae CIP *106855 (O1 El Tor)*; *V. cholerae* CIP 104151 (O139); and *V. natriegens* CIP 103193.

The National Center for Disease Control (NCDC) in Tbilisi, Georgia kindly provided two strains of *V. cholerae*: *V. cholerae* N145/P-1 (O1 classical) and *V. cholerae* N890/M-878 (O1 El Tor).

The following reference strains were obtained from the American Type Culture Collection (ATCC): *V. vulnificus* ATCC 27562; *V. parahaemolyticus* ATCC 17802; *V. metschnikovii* ATCC 700040; *V. fluvialis* ATCC 33809; *V. furnissii* ATCC 35016; *V. alginolyticus* ATCC 17749 serotype XII; *Photobacterium damselae* subsp. *damselae* ATCC 33539; *Grimontia* (*Vibrio*) *hollisae* ATCC 33564; *V. cincinnatiensis* ATCC 35912; and *V. harveyi* ATCC 14126.

Isolation of *Vibrio* Species

Different volumes of plankton-free water (10–100 mL) were passed through 0.45-μm membrane filters to concentrate bacteria. The filters were placed onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar, and incubated at 35°C for 24 h. After incubation, presumptive *Vibrio* colonies were counted. Filters were also incubated in 1% alkaline peptone water (APW), pH 8.6, for 18 h at 35°C to enhance growth of *Vibrios*. After incubation, a sterile inoculating loop was used to streak samples onto TCBS agar plates. Similarly, concentrated plankton samples were spread onto TCBS agar plates and/or inoculated in APW concentrate *Vibrios*. Plates were then incubated at 35–37°C for 24 h. Presumptive *Vibrio* colonies (yellow, green, and olive-green) that grew on TCBS plates were counted and sub-cultured onto T_1N_1 (1% trypticase, 1% sodium chloride) agar plates.

Biochemical Identification of *Vibrio* Species Isolates

Presumptive *Vibrio* isolates were sub-cultured onto Luria-Bertani agar and screened for the following biochemical properties: gelatinase production; oxidase activity; salt requirement/tolerance (growth in T_1N_1–T_1N_8 solutions); glucose oxidation/fermentation; arginine dehydrolase utilization; lysine decarboxylase utilization; and utilization of sucrose, arabinose, lactose, and mannose (4, 32).

Biochemical identification parameters were analyzed using a software algorithm designed for this study. The algorithm was used to compare biochemical tests' results for presumptive *Vibrio* isolates with those of 12 clinically important, well-characterized, standard strains of *Vibrio* species. The algorithm also included existing data on the physiological and biochemical properties of various *Vibrio* species (4, 33, 34). If the properties of a *Vibrio* isolate were similar to those of a reference strain, a percentage of affinity was calculated using different weighted factors for the particular biochemical parameter(s) (P, [P]).

Identification of *Vibrio* Species Isolated by PCR

Bacterial DNA was extracted using an AquaPure genomic DNA isolation kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Species-specific PCR (internal transcribed spacer PCR) and collagenase-targeted PCR were used to confirm the identification of presumptive *V. cholerae, V. mimicus, V. parahaemolyticus, V. alginolyticus*, and *V. vulnificus* isolates (32, 33).

To determine the serogroup (O1 or O139) of *V. cholerae* isolates, a multiplex PCR was performed as described by Huq et al. (32, 33). The assay was also used to detect virulence factor genes (e.g., ctxA) in confirmed *V. cholerae* isolates. PCR primers and target genes used to detect clinically important *Vibrio* species are listed in Table S3 in Supplementary Material.

For identification of all other presumptive *Vibrio* species not identified by species-specific PCR, 16S rRNA was amplified and sequenced, and the sequences were compared to corresponding data in NCBI and RDPII databases (33).

Direct Detection of *Vibrio* Species Isolates by PCR/ESI-MS

Vibrio species were directly detected in water samples with a *Vibrio*-specific PCR-electrospray ionization (ESI)/ mass spectrometry (MS) assay (26). Briefly, total community DNA was amplified from water samples using an eight-reaction broad-range PCR assay targeted to members of the *Vibrio* genus and *Vibrionaceae* family. After PCR, a purified aliquot from each reaction was sprayed into a Bruker Daltonics microTOF (Billerica, MA, USA) mass spectrometer. Because of the high mass accuracy (mass measurement error <1 ppm) of the spectrometer, the mass of each PCR amplicon could be accurately determined and a base composition could be assigned with confidence (e.g., xA, xT, xC, and xG) (35). Because the assay included eight primers, multiple base counts were assigned to each sample from various parts of the genome. For confirmatory identification, base compositions of the samples were added to the Ibis database (National Center for Biotechnology Information, Bethesda, MD, USA) for comparison with base compositions of *Vibrio* reference strains and related bacteria.

Direct Fluorescent Antibody Test

A direct fluorescent antibody (DFA) test was used to detect *V. cholerae* O1 and O139 in enriched and concentrated water and plankton samples (32, 33). Preparation of specimens was performed using a DFA test kit (New Horizon Diagnostics, Columbia, MD, USA) according to the manufacturer's instructions. Specimens were examined with an epi-fluorescent microscope (Axioskop 40, Opton Zeiss, Germany) at 100× magnification.

Data Analysis

Each water sample was tested in triplicate for all biochemical tests outlined above, and the mean values and standard errors were calculated for each variable on a given sampling date. For the purpose of analysis, seasons were defined as follows:

- *Winter*: December through February;
- *Spring*: March through May;
- *Summer*: June through August;
- *Fall*: September through November.

Statistical analysis was carried out using the Statistical Toolpak for Microsoft Excel 2010. Correlations (Pearson's *r*) between factors described herein were significant at the 0.05 level.

RESULTS AND DISCUSSION

Isolation and Phenotypic Characterization of Clinically Important *Vibrio* Species

During the study period, 1,440 water samples were collected and analyzed; from these, 1,595 presumptive *Vibrio* isolates were collected. Among these samples, 657 presumptive *Vibrio* isolates were collected from four sites on the Black Sea coast and 938 from three inland water reservoirs. Over 70% of the isolates were assigned to 10 clinically important *Vibrio* species based on biochemical characteristics. These isolates were recovered from concentrated and enriched water samples, as well as from plankton. Of the 1,595 isolates, 856 were identified as presumptive non-halophilic *Vibrios* (specifically, *V. cholerae* and *V. mimicus*) and 739 were identified as halophilic *Vibrio* species. As expected, the Black Sea *Vibrio* population was most the diverse: nine species of clinically important *Vibrios* were recovered (*V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *V. metschnikovii*, *V. alginolyticus*, *V. harveyi*, *V. furnissii*, *V. fluvialis*, and *V. cincinnatiensis*). Only seven *Vibrio* species (*V. cholerae*, *V. vulnificus*, *V. mimicus*, *V. metschnikovii*, *V. alginolyticus*, *V. harveyi*, and *V. fluvialis*) were collected from the inland water reservoirs. In general, halophilic *Vibrio* species were more commonly found in the Black Sea coastal zones (47.8%), than in freshwater lakes (6.2%). Among the halophilic *Vibrio* species, *V. parahaemolyticus* was the most prevalent. *V. cholerae* comprised more than 65% of isolates from inland reservoirs, and 23% of those from Black Sea sites. It should be noted that up to 28% of isolates were identified only to genus level. In a separate study by Mitaishvili et al., some of these isolates were identified as non-human-pathogenic *Vibrio* species, including *V. natriegens*, *V. splendidus*, and *V. estuarinus* (36).

PCR Identification of *Vibrio* Species

To establish the diversity of the *Vibrio* species more accurately and also for comparison purposes, a subset of 274 halophilic *Vibrio* isolates (attributed to eight *Vibrio* species, mostly *V. parahaemolyticus*) and 520 non-halophilic *Vibrio* isolates (512 *V. cholerae* and 8 *V. mimicus*) were subjected to either PCR with species-specific primers; or amplification, sequencing, and comparison of 16S rRNA signatures, employing data available in public databases. A high level of agreement (98.5%, or 503 isolates out of 512) was observed between standard biochemical and PCR identifications for *V. cholerae*. Similar results were observed among 144 isolates of *V. parahaemolyticus* (95.8%).

The agreement between the two methods was relatively low (38.5–46.5%) for other *Vibrio* species. For example, some of the *V. vulnificus* isolates presumptively identified based on biochemical analysis were found to be *V. cholerae* by PCR. The majority of presumptive *V. alginolyticus* and *V. cincinnatiensis* isolates were identified as *V. parahaemolyticus* by PCR. Additionally, several isolates of *V. fluvialis identified by biochemical tests* were determined to be *V. furnissii* by genetic analysis. These results most likely are due to the significant genetic variability of biochemical features (e.g., salt tolerance, sucrose, lactose, and arabinose utilization) and especially of the halophilic *Vibrio* species.

Among 794 presumptive *Vibrio* isolates, 10 of the clinically important *Vibrio* species were genetically identified. The distribution of species identified by biochemical analysis was different than that indicated by genetic testing. Among PCR-confirmed *Vibrio* isolates, *V. cholerae* was most prevalent (64.6%) among tested isolates, followed by *V. parahaemolyticus* (21.4%), *V. vulnificus* (4.0%), and *V. alginolyticus* (2.6%). Combined, the remaining six species (*V. metschnikovii*, *V. harveyi*, *V. furnissii*, *V. fluvialis*, *V. cincinnatiensis*, and *V. mimicus*) accounted for 4.4% of samples. Genetic identification was not definitive for 3% of the *Vibrio* isolates. The distributions of the 10 *Vibrio* species confirmed by PCR are presented in **Figure 1**.

Direct Detection of Clinically Important *Vibrio* Species in Water Samples

In parallel with culture isolation, *Vibrio* species were directly detected in water samples collected during 2006–2008 from marine and freshwater sites ($n = 440$ samples; 220 from the Black Sea and 220 from lakes) using a PCR/ESI-MS method verified by Whitehouse et al. in a previous study employing standard and environmental *Vibrio* strains (26). Eight clinically important *Vibrio* species were detected by this method. Six of the eight *Vibrio* species were halophilic (*V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. harveyi*, *V. metschnikovii*, and *V. cincinnatiensis*); the remaining two *Vibrio* species were non-halophilic (*V. cholerae* and *V. mimicus*), that is, not requiring additional salt for growth (all *Vibrios* have an absolute requirement for NaCl for growth). Two non-pathogenic *Vibrio* species [*V. natriegens* and *V. (Comamonas) neocistes*] were also detected.

The distribution of samples positive for specific *Vibrio* species by PCR/ESI-MS is presented in **Figure 2**. In alignment with culture results, *V. cholerae* was the most prevalent pathogenic *Vibrio* species. In water samples containing the DNA of at least one *Vibrio* species, 62.8% contained *V. cholerae* ($n = 304$).

Population diversity of Black Sea *Vibrio* species as determined by PCR/ESI-MS was similar to that observed among biochemically identified species (**Figure 3A**). Halophilic *Vibrio* species were more abundant in samples collected at Black Sea sites (72.6% of all *Vibrio*-positive samples) than those from freshwater lakes (29.1%); this is in agreement with culture results. Among *Vibrio*-positive Black Sea water samples ($n = 153$), *V. parahaemolyticus* was most frequently detected (53.6%), followed by *V. cholerae* and *V. vulnificus* (48.4 and 36.6%, respectively). Other *Vibrio* species (*V. alginolyticus, V. metschnikovii, V. fluvialis,* and *V. harveyi*) comprised between 0.7 and 3.9% of those identified in Black Sea samples.

Of the seven *Vibrio* species identified by conventional culture, six were detected in fresh and brackish lake samples, using PCR/ESI-MS (**Figure 3B**). *V. cholerae* was most frequently isolated from water samples collected at all three inland reservoirs (77.5% of all positive samples). DNA from other *Vibrio* spp. was detected less frequently, including: *V. parahaemolyticus* (21.2%), *V. mimicus* (6.0%), *V. metschnikovii* (4.6%), and *V. vulnificus* (2.0%). Among the lake samples, abundance and diversity of *Vibrio* species were highest in Kumisi Lake, with six species identified by phenotypic characterization and five by PCR. This diversity is most likely related to the relatively high salinity of the lake.

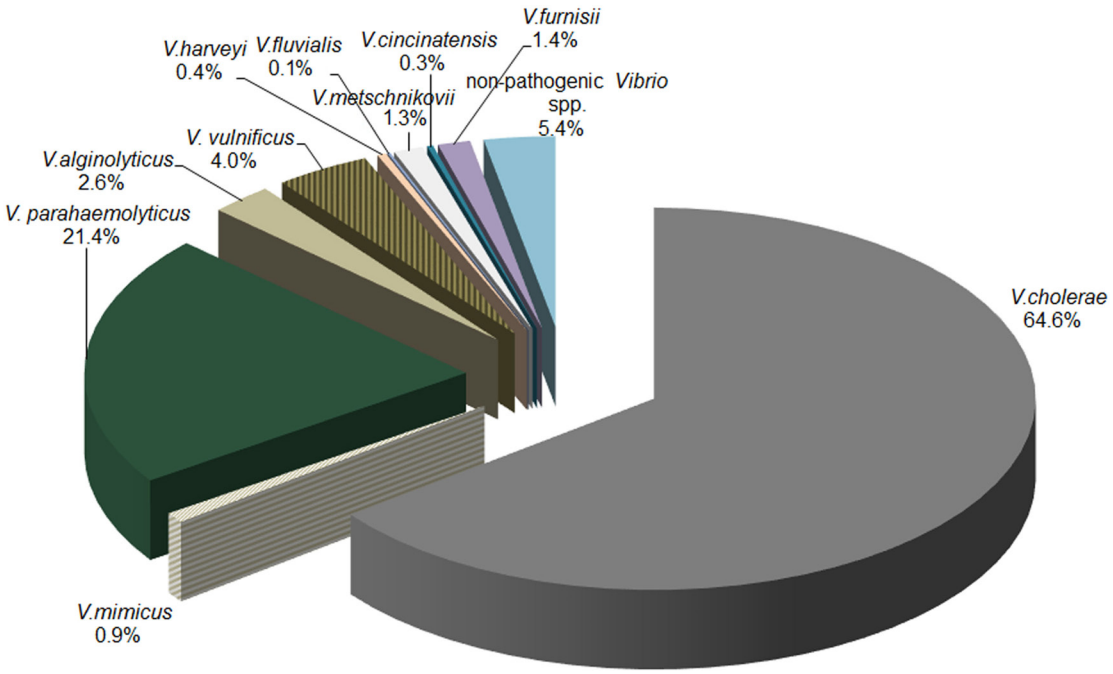

FIGURE 1 | Distribution of *Vibrio* species via PCR. The distribution of *Vibrio* species detected by PCR in water samples collected across Georgia.

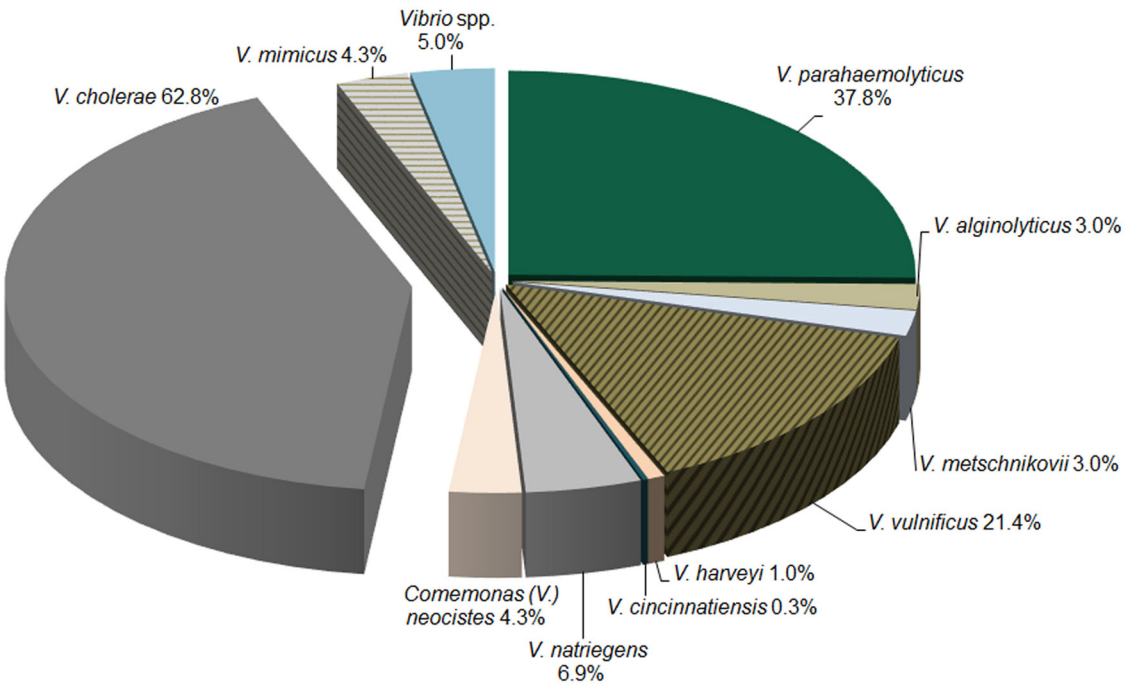

FIGURE 2 | Distribution of *Vibrio* species via PCR/ESI-MS. The distribution of *Vibrio* species detected by PCR/ESI-MS in water samples collected across Georgia.

Also, in agreement with results of our previously published study, 20.7% of Black Sea and lake samples contained *V. natriegens* and/or *V. neocistes*. Also as observed in our previous report, *V. natriegens* was cultured from all four Black Sea sites along with other *Vibrio* species that are not pathogenic for humans (36).

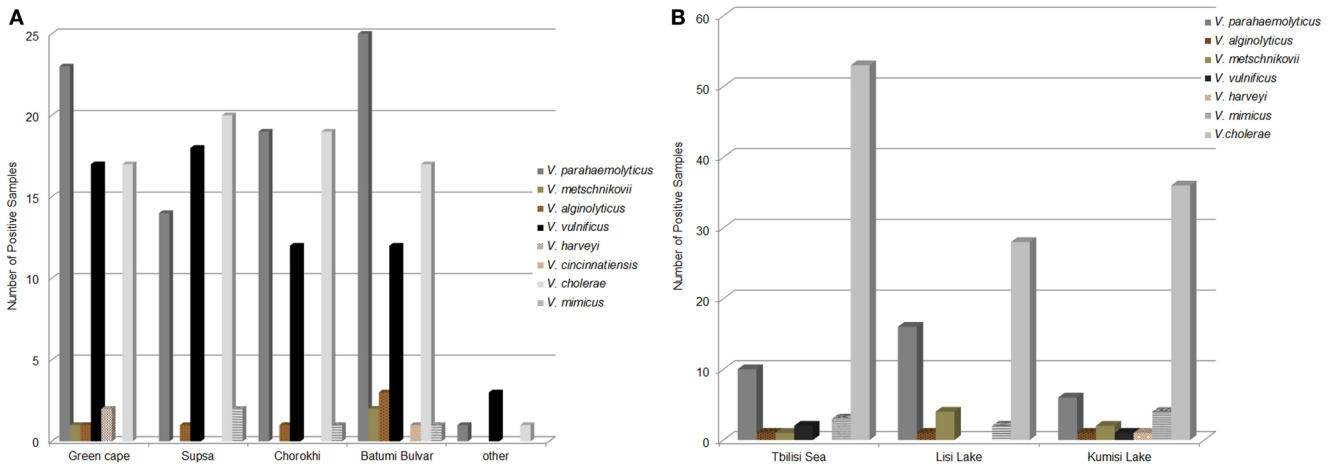

FIGURE 3 | Abundance of *Vibrio* species in Black Sea and lake water samples. The abundance of *Vibrio* species in water samples as determined by PCR/ESI-MS from **(A)** The Black Sea and **(B)** Freshwater and brackish lakes.

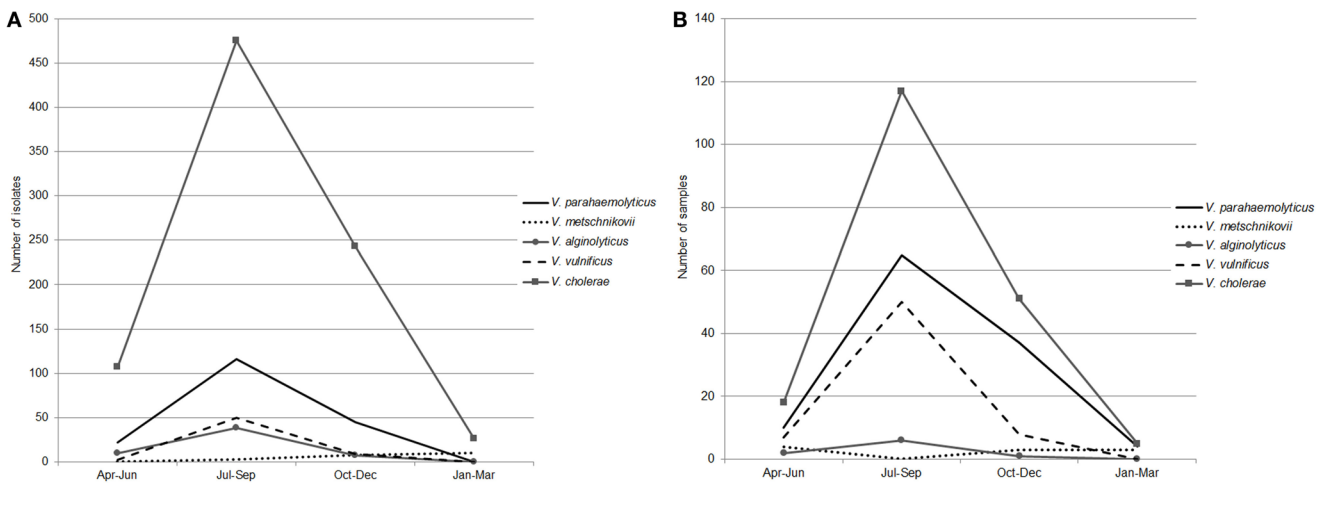

FIGURE 4 | Seasonal abundance of five *Vibrio* species. The seasonal abundance of five *Vibrio* species (*V. parahaemolyticus*, *V. metschnikovii*, *V. alginolyticus*, *V. vulnificus*, and *V. cholerae*) as determined by **(A)** Biochemical identification and, **(B)** PCR/ESI-MS. Seasonal water temperature is reported in Table S2 in Supplementary Material.

Seasonality in Abundance of Clinically Important *Vibrio* Species

Seasonal patterns in abundance of clinically important *Vibrio* species were reflected in the isolation frequency and results of PCR/ESI-MS. Ninety-five percent of *Vibrio* spp. were isolated from April through November, with the highest detection rates in July, August, and September (**Figures 4A,B**); water temperatures for these time periods (per location) are reported in Table S2 in Supplementary Material. In our earlier reports, we described a positive correlation between total *Vibrio* counts and water temperature at Black Sea and inland lake sites (37, 38). In addition, in the current study, we observed a correlation between isolation frequency of distinct groups of *Vibrio* species and water temperature at marine and freshwater sites. Interestingly, in this study, the correlation coefficient was higher for halophilic *Vibrio* species ($r = 0.85$ for Black Sea sites and $r = 0.74$ for lakes) than for *V. cholerae* ($r = 0.45$ for Black Sea sites and $r = 0.64$ for lakes). A different pattern of seasonal abundance was observed for *V. metschnikovii* isolates, which were collected during the cold season and were detected by PCR/ESI-MS from October through April (**Figures 4A,B**). This is indicative of cold tolerance of *V. metschnikovii* in comparison with the other pathogenic *Vibrio* species (39, 40).

Salinity as an Environmental Factor Influencing *Vibrio* Community Composition

Salinity, an important environmental factor, can influence microbial community composition and abundance in aquatic

environments (1, 2, 7). In an earlier published study, we reported no significant correlation between total *Vibrio* counts and salinity for Black Sea samples; instead, species diversity (by culturing and direct detection techniques) appears to be linked to salinity (37). For example, PCR/ESI-MS direct detection (**Figure 3A**) showed less diversity among *Vibrio* species in the Supsa and Chorokhi estuaries (five species at each site) than at the Green Cape and Batumi Boulevard sites (six and seven species, respectively). In the samples collected from Batumi Boulevard sites where the salinity is higher (16–20%), *V. parahaemolyticus* DNA was detected more frequently by PCR/ESI-MS than in samples from the Supsa estuary (salinity 3–10.6%), although a similar pattern of seasonal temperature dependence was observed at both Black Sea sampling stations (**Figures 5A,B**).

Among inland reservoirs, a more diverse *Vibrio* population was detected by both culture and PCR/ESI-MS in Kumisi Lake (five clinically important *Vibrio* species in total) than the other two lakes (**Figure 3B**). It was surprising to detect a number of pathogenic halophilic *Vibrio* species in inland lakes, where non-halophilic *Vibrios* were predicted (and ultimately isolated) to be more prevalent; for example, *V. alginolyticus* was detected in these reservoirs, and was most abundant in the Tbilisi Sea (average salinity of 0.1%). This may be explained by the proximity of this artificially created reservoir (fed by the Iori River) to three small saltwater lakes that existed in the area before the creation of the Tbilisi Sea in the 1950s (41).

V. cholerae: A Prevalent Human-Pathogenic *Vibrio* in Georgian Aquatic Environments

The most important human-pathogenic *Vibrio* species, *V. cholerae*, was demonstrated by culture and direct detection to be prevalent in Georgian aquatic environments. According to PCR/ESI-MS, *V. cholerae* was present in the majority of lake samples (77.5% of positive samples). *V. cholerae* was detected less frequently in Black Sea samples (48.4%). This aligned with culture results, among which *V. cholerae* comprised 23 and 75.2% of *Vibrio* species isolates from the Black Sea and lake samples, respectively. According to our earlier report, the vast majority (94.6%) of Georgian isolates of *V. cholerae* were attributed to the non-O1/non-O139 group (28). These groups are generally non-pathogenic to humans, although some serotypes can cause mild to severe gastroenteritis (8, 9).

Epidemic *V. cholerae* serotype O1 was directly detected by DFA in the concentrated and enriched water and plankton samples collected at all four sites in the coastal zone of the Black Sea during the study period (**Figure 6**). Toxigenic *V. cholerae* O1 was also detected in lake samples (specifically from Kumisi Lake and the Tbilisi Sea) collected in 2008 and 2009 (**Figure 6**). In addition, signals indicative of *V. cholerae* O139 were detected in eight marine samples collected in the summer of 2008. These data, in combination with results of our earlier DFA investigations on lake samples collected in 2006 and 2007, indicate that toxigenic *V. cholerae* is common in all of the target water bodies in this study (25). These serotypes were detected most frequently in Black Sea samples and often associated with plankton fractions (**Figure 7**). This is in agreement with previously published serological data and multiplex PCR data from other investigators studying *V. cholerae* (28). Forty-six isolates (most of which were collected from enriched marine samples) were confirmed as *V. cholerae* serotype O1 and six isolates were presumptively identified as *V. cholerae* serotype O139. Some of the *V. cholerae* O1 strains belonged to the El Tor biotype, while others revealed characteristics of hybrid variants. Two O1 and six non-O1 strains carried ctx genes.

Interestingly, *V. cholerae* was observed along with two other important *Vibrio* species in the same ecological niche: *V. parahaemolyticus* (33.1% of PCR-ESI/MS samples) and *V. vulnificus* (11.6%). The increased probability of co-mingling between *V. cholerae* and these important *Vibrio* species could facilitate transfer of genetic material (e.g., virulence factors) between the species.

Comparative Ecology of *V. parahaemolyticus* in Marine and Lake Reservoirs

The second most important human-pathogenic *Vibrio* species, *V. parahaemolyticus*, was detected by PCR/ESI-MS in samples from the Black Sea. Most surprisingly, *V. parahaemolyticus* was detected in all sampled lakes, but no confirmed *V. parahaemolyticus* isolates were recovered from these sites. Interestingly, *V. parahaemolyticus* was more frequently detected by PCR/ESI-MS in concentrated lake samples than enriched lake samples; the reverse of this was observed for Black Sea samples (**Figure 8**). For comparison, as expected, DNA from *V. cholerae* was found most frequently in enriched lake samples, and in equal quantities in enriched and concentrated marine samples. The abundance of *V. parahaemolyticus* DNA in fresh and brackish water sites is likely explained by the lower salinity of lake water, which is a stressor for *V. parahaemolyticus*, a moderately halophilic bacterium. The low frequency of isolation of *V. parahaemolyticus* from lake water, coupled with the relatively high frequency of direct molecular detection, suggests that the species may be present in a viable but non-culturable state. Isolation of *V. parahaemolyticus*-specific bacteriophages from Lake Kumisi and Lake Lisi samples also supports the conclusion that this *Vibrio* species is present (42). The possibility of *V. parahaemolyticus* existing in a transitional state in freshwater reservoirs, in association with freshwater fish, has been proposed by Sarkar et al. and other researchers and is important, as this may serve as a potential exposure route to the bacterium (43, 44).

CONCLUSION

In this study, we describe the occurrence, diversity, and seasonal distribution of clinically important *Vibrio* species in recreational waters in the South Caucasus. In total, 10 clinically important *Vibrio* species (of 12 known species) were detected among these sites; nine of which were detected in Black Sea coastal waters and six in inland sites. The Black Sea *Vibrio* populations were dominated by *V. parahaemolyticus*, followed by *V. cholerae*, *V. vulnificus,* and *V. alginolyticus*. *V. cholerae* was

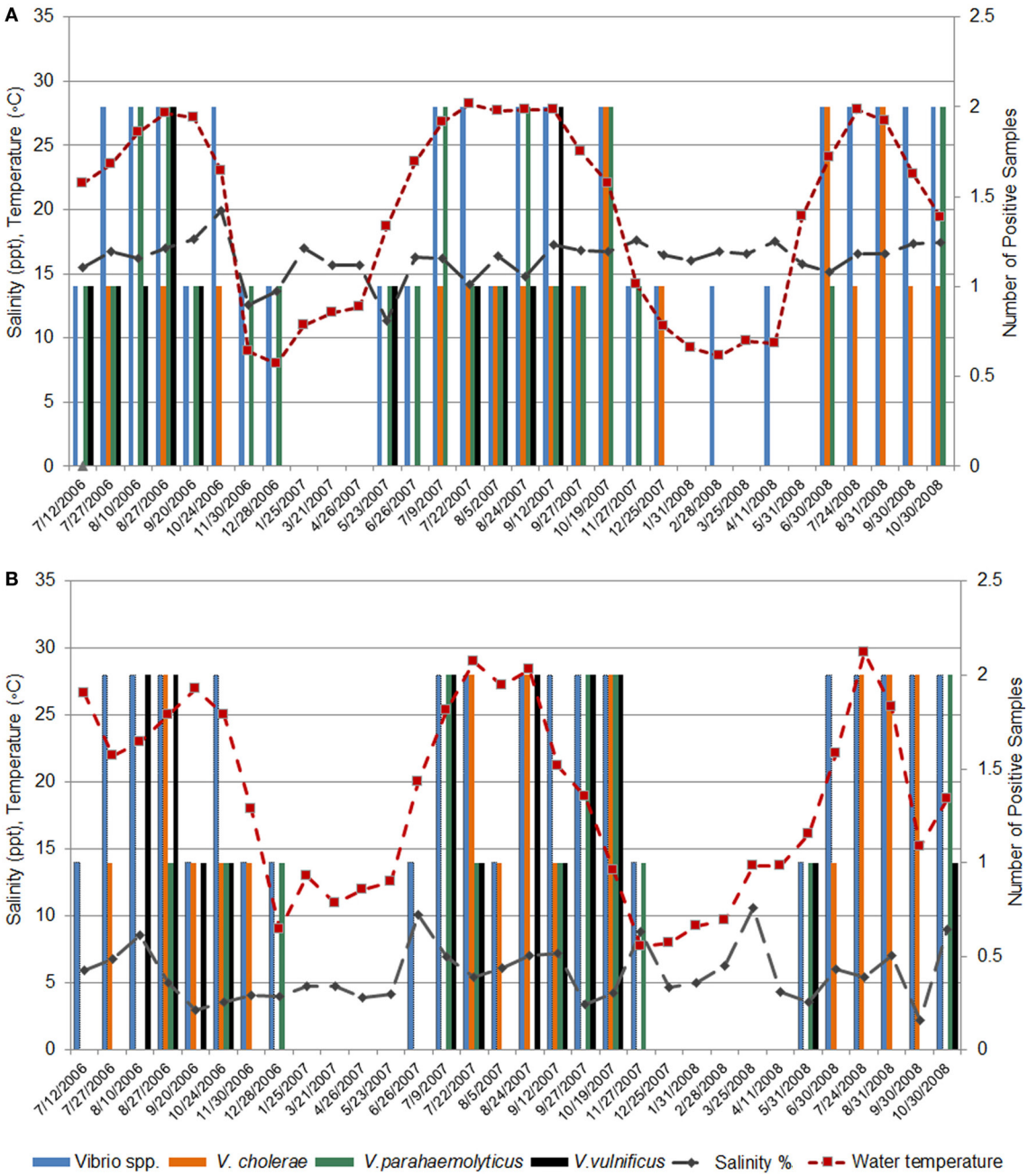

FIGURE 5 | Influence of water temperature on the detection of *Vibrio* species. The influence of water temperature on the overall detection rates of *Vibrio* species by PCR/ESI-MS for **(A)** Batumi Boulevard, **(B)** Supsa estuary.

readily detected in fresh and brackish water samples collected at sites near Tbilisi.

V. cholerae was observed in the same ecological niche as two other important *Vibrio* species: *V. vulnificus* and *V. alginolyticus*. This finding is significant for several reasons. First, the possibility of acquiring infections caused by these pathogens will likely increase as the concentration/diversity of pathogenic *Vibrio* species located in a single niche increases. Second, such cohabitation increases the opportunity for interspecies interactions and potential exchanges of genetic material such as virulence factor genes.

As observed in previous studies, isolation of target *Vibrio* species and their direct detection using DNA-based methods for detection in marine and lake samples revealed seasonal patterns related to temperature that suggested that temperature affects *Vibrio* abundance, while salinity affects *Vibrio* species composition (37, 38).

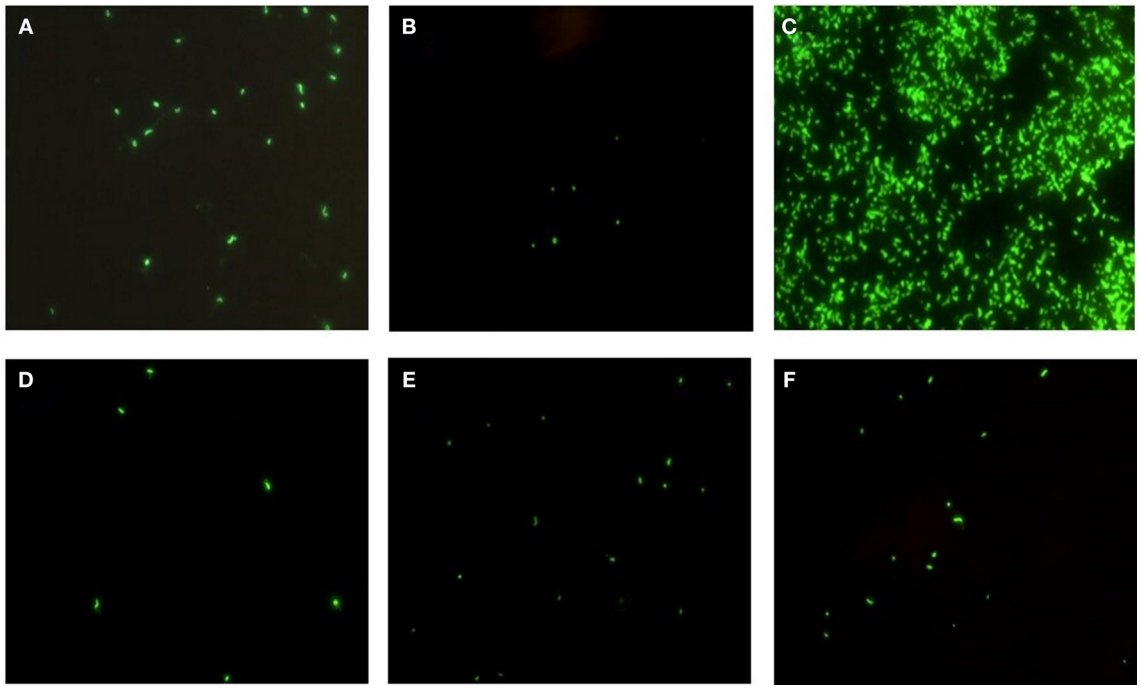

FIGURE 6 | Detection of *V. cholerae* O1 by DFA in Black Sea and lake samples. Detection of *V. cholerae* O1 in water samples from **(A–C)** Green Cape sample sites on the Black Sea, and **(D–F)** Kumisi Lake. Concentrated water samples are shown in **(A,D)**, plankton samples in **(B,E)** and enriched water samples in **(C,F)**. Organisms were detected and visualized using a direct fluorescent-monoclonal antibody (DFA) kit.

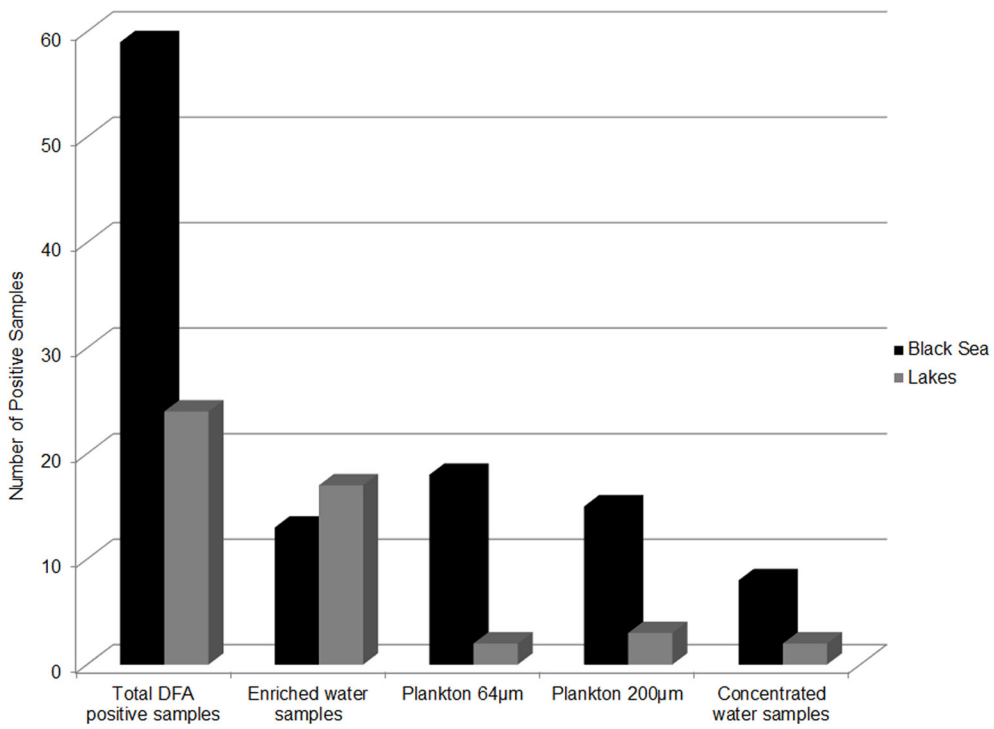

FIGURE 7 | Distribution of *V. cholerae* O1 DFA positive samples. The distribution by sample type and sampling site of *V. cholerae* O1 positive samples as detected by DFA.

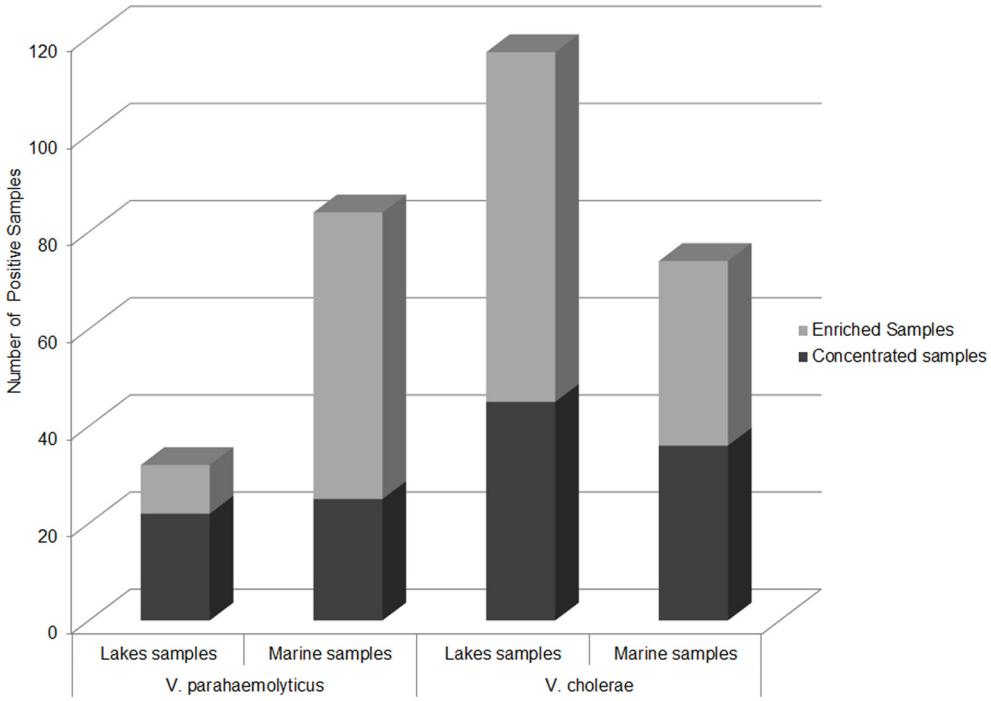

FIGURE 8 | Abundance of PCR/ESI-MS positive samples of *V. parahaemolyticus* and *V. cholerae*. Number of samples by sample type and sample site that yielded detectable levels of *V. parahaemolyticus* and *V. cholerae* via PCR/ESI-MS.

Data obtained by conventional bacteriology, DFA, and PCR/ESI-MS analysis indicate that environmental reservoirs for toxigenic *V. cholerae* O1 (possibly O139 at some marine sites) are present in the recreational waters of Georgia, including freshwater lakes near Tbilisi, and the Georgian coastal zone of the Black Sea. Of particular note is the detection of *V. cholerae* O1 in the Tbilisi Sea, a freshwater reservoir that serves as a source of drinking water for several districts of Tbilisi. Previous detection of pandemic variants of *V. parahaemolyticus* (serotypes O3:K6) suggested increased risk for infection with the organism in areas where it is detected but not known to have (or that are not monitored for) outbreaks of seafood-related gastroenteritis or other water-related illnesses (28). This is even more apparent when the high frequency of seasonally reported enteric diseases with idiopathic etiologies in Georgia (especially in the Ajara region) is taken into account (30, 31).

Perpetually changing environmental conditions (e.g., increasing surface water temperatures) can significantly elevate the risk of infections related to potentially human-pathogenic *Vibrio* species. Such changes may also affect temperate regions with mild subtropical climates such as the South Caucasus. In the South Caucasus, climate change may lead to accelerated ecological problems and raise additional public health issues. In the coming decade, increases in air temperature (with associated long-lasting hot summers) and frequent heavy storms, combined with elevated surface water temperatures (up to ≥30°C) predicted for the Black Sea coastal areas, could trigger massive mortalities of aquatic organisms and possibly a significant rise in the incidence of diarrheal disease (30). Similarly, we can expect to observe such effects of climate change (with corresponding consequences for public health) associated with the recreational water bodies around Tbilisi.

In summary, although the occurrence of potentially pathogenic autochthonous water bacteria such as *Vibrios* cannot be controlled in natural ecosystems, the likelihood of the incidence of human illness can be reduced. This can be accomplished by limiting exposure to recreational water bodies with suspected elevated numbers of pathogenic *Vibrio* species. Furthermore, exposure can also be limited in areas where changes in ecological parameters have the potential to trigger proliferation of clinically important *Vibrio* species. Therefore, regular monitoring of water reservoirs for possible microbial pathogens is recommended to allow for early response by public health authorities (e.g., prevention and treatment measures to combat relevant diseases). The results presented in this report, as well as those in our previous publications (17, 27, 30) provide a foundation for effective monitoring of target *Vibrio* species, as well as for creation of predictive models based on environmental indicators.

ACKNOWLEDGMENTS

This research was made possible by financial support provided by the U.S. Department of Defense (DoD), Defense Threat Reduction Agency (DTRA), through the Cooperative Biological Engagement Program's (CBEP) Cooperative Biological Research Project (CBR) GG-13, in Georgia. The research was also partially supported by the National Science Foundation grant

(No. 0813066) and the National Institutes of Health grant (No. 2RO1A1039129-11A2) awarded to Drs. Anwar Huq and Rita Colwell of the University of Maryland. The findings, opinions, and views expressed herein belong to the authors and do not reflect an official position of any organization listed. The authors are grateful to George Mikuchadze for his assistance in algorithm development for biochemical identification of *Vibrio* species. The authors also thank Attimo Research and Development, and Sonya Narodny for editorial assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpubh.2015.00232

REFERENCES

1. Belkin S, Colwell RR. *Oceans and Health: Pathogens in the Marine Environment*. New York, NY: Springer (2005). 474 p.
2. Thompson FL, Iida T, Swings J. Biodiversity of *Vibrios*. *Microbiol Mol Biol Rev* (2004) **68**(3):403–31. doi:10.1128/MMBR.68.3.403-431.2004
3. Barbieri E, Falzano L, Fiorentini C, Pianetti A, Baffone W, Fabbri A, et al. Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Appl Environ Microbiol* (1999) **65**(6):2748–53.
4. Farmer JJ, Janda J, Birkhead K. *Vibrio: Manual of Clinical Microbiology*. 8 ed. Washington, DC: ASM Press (2003). p. 706–18.
5. Johnson CN, Bowers JC, Griffitt KJ, Molina V, Clostio RW, Pei S, et al. Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). *Appl Environ Microbiol* (2012) **78**(20):7249–57. doi:10.1128/AEM.01296-12
6. Hlady WG, Klontz KC. The epidemiology of *Vibrio* infections in Florida, 1981-1993. *J Infect Dis* (1996) **173**(5):1176–83. doi:10.1093/infdis/173.5.1176
7. Pfeffer CS, Hite MF, Oliver JD. Ecology of *Vibrio vulnificus* in estuarine waters of eastern North Carolina. *Appl Environ Microbiol* (2003) **69**(6):3526–31. doi:10.1128/AEM.69.6.3526-3531.2003
8. Morris JG Jr. Cholera – modern pandemic disease of ancient lineage. *Emerg Infect Dis* (2011) **17**(11):2099–104. doi:10.3201/eid1711.111109
9. Onishenko G, Ganin V, Golubinsky E. *Vibrios of Non-O1 Serological Group and Their Importance in Human Pathology*. Moscow: Ministry of Health of the Russian Federation (2001).
10. Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, et al. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J Clin Microbiol* (1997) **35**(12):3150–5.
11. DePaola A, Ulaszek J, Kaysner CA, Tenge BJ, Nordstrom JL, Wells J, et al. Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. *Appl Environ Microbiol* (2003) **69**(7):3999–4005. doi:10.1128/AEM.69.7.3999-4005.2003
12. Varghese MR, Farr RW, Wax MK, Chafin BJ, Owens RM. *Vibrio fluvialis* wound infection associated with medicinal leech therapy. *Clin Infect Dis* (1996) **22**(4):709–10. doi:10.1093/clinids/22.4.709a
13. Hickman FW, Farmer JJ, Hollis DG, Fanning GR, Steigerwalt AG, Weaver RE, et al. Identification of *Vibrio hollisae* sp. nov. from patients with diarrhea. *J Clin Microbiol* (1982) **15**(3):395–401.
14. Uh Y, Park JS, Hwang GY, Jang IH, Yoon KJ, Park HC, et al. *Vibrio alginolyticus* acute gastroenteritis: report of two cases. *Clin Microbiol Infect* (2001) **7**(2):104–6. doi:10.1046/j.1469-0691.2001.00207.x
15. Hornstrup MK, Gahrn-Hansen B. Extraintestinal infections caused by *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in a Danish county, 1987-1992. *Scand J Infect Dis* (1993) **25**(6):735–40. doi:10.3109/00365549309008571
16. Shin JH, Shin MG, Suh SP, Ryang DW, Rew JS, Nolte FS. Primary *Vibrio damsela* septicemia. *Clin Infect Dis* (1996) **22**(5):856–7. doi:10.1093/clinids/22.5.856
17. Nishibushi M. Miscellaneous human pathogens. In: Thompson F, Austin B, Swings J, editors. *The Biology of Vibrios*. ASM Press (2006). 423 p.
18. Chatterjee SH, Haldar S. *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods. *J Marine Sci Res Dev* (2012) **S1**:002. doi:10.4172/2155-9910.S1-002
19. Cavallo RA, Stabili L. Culturable *Vibrios* biodiversity in the Northern Ionian Sea (Italian coasts). *Sci Mar* (2004) **68**(S1):23–9. doi:10.3989/scimar.2004.68s123
20. Danovaro R, Fonda Umani S, Pusceddu A. Climate change and the potential spreading of marine mucilage and microbial pathogens in the Mediterranean Sea. *PLoS One* (2009) **4**(9):e7006. doi:10.1371/journal.pone.0007006
21. Cantet F, Hervio-Heath D, Caro A, Le Mennec C, Monteil C, Quemere C, et al. Quantification of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* in French Mediterranean coastal lagoons. *Res Microbiol* (2013) **164**(8):867–74. doi:10.1016/j.resmic.2013.06.005
22. Aldova E, Zakhariev ZA, Dinev TS, Zlatanov ZT. [*Vibrio parahaemolyticus* in the Black Sea]. *Zentralbl Bakteriol Orig* (1971) **218**(2):176–88.
23. Libinzon AE, Domaradskii IV, Us ZI, Demina AI, Nagornaia AF. [Parahemolytic *Vibrios* and related halophilic microorganisms of the Black Sea]. *Zh Mikrobiol Epidemiol Immunobiol* (1974) **00**(5):80–4.
24. Libinzon AE, Gal'tseva GV. [Taxonomic position of representatives of the species *Vibrio cholerae*]. *Zh Mikrobiol Epidemiol Immunobiol* (1981) **11**:41–4.
25. Grim CJ, Jaiani E, Whitehouse CA, Janelidze N, Kokashvili T, Tediashvili M, et al. Detection of toxigenic *Vibrio cholerae* O1 in freshwater lakes of the former Soviet Republic of Georgia. *Environ Microbiol Rep* (2010) **2**(1):2–6. doi:10.1111/j.1758-2229.2009.00073.x
26. Whitehouse CA, Baldwin C, Sampath R, Blyn LB, Melton R, Li F, et al. Identification of pathogenic *Vibrio* species by multilocus PCR-electrospray ionization mass spectrometry and its application to aquatic environments of the former Soviet Republic of Georgia. *Appl Environ Microbiol* (2010) **76**(6):1996–2001. doi:10.1128/aem.01919-09
27. Akhmedov R, Haley BJ, Rajabov M, Ahmadova S, Gurbanov S, Colwell RR, et al. Detection of *Vibrio cholerae* in environmental waters including drinking water reservoirs of Azerbaijan. *Environ Microbiol Rep* (2013) **5**(1):30–8. doi:10.1111/j.1758-2229.2012.00369.x
28. Kokashvili T, Elbakidze T, Jaiani E, Janelidze N, Kamkamidze G, Whitehouse C, et al. Comparative phenotypic characterization of *Vibrio cholerae* isolates collected from aquatic environments of Georgia. *Georgian Med News* (2013) **224**:55–62.
29. Haley BJ, Kokashvili T, Tskshvediani A, Janelidze N, Mitaishvili N, Grim CJ, et al. Molecular diversity and predictability of *Vibrio parahaemolyticus* along the Georgian coastal zone of the Black Sea. *Front Microbiol* (2014) **5**:45. doi:10.3389/fmicb.2014.00045
30. UND Programme. *Climate Change Strategy of Ajara*. Tbilisi: The United Nations Development Programme (2013).
31. Ministry of Labor H, Social Affairs of Georgia. Health care: statistical yearbook. Georgia 2010. In: NCfDCaP, editor. *Health*. Atlanta, GA: Department of Medical Statistics of National Centre for Disease Control and Public Health (2011) 75–87.
32. Huq A, Grim C, Colwell RR, Nair GB. Detection, isolation, and identification of *Vibrio cholerae* from the environment. *Curr Protoc Microbiol* (2006) **Chapter 6**:6A.5. doi:10.1002/9780471729259.mc06a05s02
33. Huq A, Haley BJ, Taviani E, Chen A, Hasan NA, Colwell RR. Detection, isolation, and identification of *Vibrio cholerae* from the environment. *Curr Protoc Microbiol* (2012) **Chapter 6**:6A.5. doi:10.1002/9780471729259.mc06a05s26
34. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. *Bergey's Manual of Determinative Bacteriology*. Baltimore, MD: Williams & Wilkins (1994).
35. Muddiman DC, Anderson GA, Hofstadler SA, Smith RD. Length and base composition of PCR-amplified nucleic acids using mass measurements from electrospray ionization mass spectrometry. *Anal Chem* (1997) **69**(8):1543–9. doi:10.1021/ac961134r

36. Mitaishvili N, Tskhvediani A, Elbakidze T, Kokashvili T, Natroshvili G, Kajaia G, et al. The abundance and diversity of *Vibrio* species not pathogenic to humans Georgian aquatic environment. *Proc Georgian Acad Sci* (2013) **9**:43–9.
37. Janelidze N, Jaiani E, Lashkhi N, Tskhvediani A, Kokashvili T, Gvarishvili T, et al. Microbial water quality of the Georgian coastal zone of the Black Sea. *Mar Pollut Bull* (2011) **62**(3):573–80. doi:10.1016/j.marpolbul.2010.11.027
38. Jaiani E, Kokashvili T, Mitaishvili N, Elbakidze T, Janelidze N, Lashkhi N, et al. Microbial water quality of recreational lakes near Tbilisi, Georgia. *J Water Health* (2013) **11**(2):333–45. doi:10.2166/wh.2013.057
39. Lee J, Donovan T, Furniss AL. Characterization, taxonomy, and emended description of *Vibrio metschnikovii*. *Int J Syst Bacteriol* (1978) **28**(1):99–111. doi:10.1099/00207713-28-1-99
40. Arunagiri K, Jayashree K, Sivakumar T. Isolation and identification of *Vibrios* from marine food resources. *Int J Curr Microbiol App Sci* (2013) **2**(7):217–32.
41. Abchazava IS. *The Lakes of Georgia*. Tbilisi: Metsniereba (1975).
42. Tskhvediani A, Khukhunashvili T, Porchkhidze K, Tsertsvadze G, Tediashvili M. The possible use of *V. parahaemolyticus* – specific bacteriophages for prevention and therapy of infections caused by *V. parahaemolyticus*. *Georgian Med News* (2014) **6**(231):82–8.
43. Sarkar BL, Nair GB, Banerjee AK, Pal SC. Seasonal distribution of *Vibrio parahaemolyticus* in freshwater environs and in association with freshwater fishes in Calcutta. *Appl Environ Microbiol* (1985) **49**(1):132–6.
44. Noorlis A, Ghazali FM, Cheah YK, Tuan Zainazor TC, Ponniah J, Tunung R, et al. Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. *Int Food Res J* (2011) **18**:689–95.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 10 September 2015
doi: 10.3389/fmicb.2015.00917

Prevalence and distribution of soil-borne zoonotic pathogens in Lahore district of Pakistan

Muhammad Z. Shabbir[1], Tariq Jamil[1], Asad A. Ali[1], Arfan Ahmad[1], Muhammad Naeem[2], Muhammad H. Chaudhary[3], Muhammad Bilal[1], Muhammad A. Ali[1], Khushi Muhammad[1], Tahir Yaqub[1], Asghari Bano[2], Ali I. Mirza[4], Muhammad A. B. Shabbir[1], Walter R. McVey[5], Ketan Patel[6], Stephen Francesconi[6], Bhushan M. Jayarao[5] and Masood Rabbani[1]*

[1] University of Veterinary and Animal Sciences, Lahore, Pakistan, [2] Quaid-i-Azam University, Islamabad, Pakistan, [3] University of the Punjab, Lahore, Pakistan, [4] Government College University, Lahore, Pakistan, [5] Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA, USA, [6] Naval Medical Research Unit, Frederick, MA, USA

OPEN ACCESS

Edited by:
Jeanne Marie Fair, Defense Threat Reduction Agency, USA

Reviewed by:
Li Xu, Cornell University, USA
Chris Yeager, Los Alamos National Laboratory, USA

***Correspondence:**
Bhushan M. Jayarao, Penn State Animal Diagnostic Laboratory, Wiley Lane, University Park, PA 16802, USA
bmj3@psu.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *21 May 2015*
Accepted: *21 August 2015*
Published: *10 September 2015*

Citation:
Shabbir MZ, Jamil T, Ali AA, Ahmad A, Naeem M, Chaudhary MH, Bilal M, Ali MA, Muhammad K, Yaqub T, Bano A, Mirza AI, Shabbir MAB, McVey WR, Patel K, Francesconi S, Jayarao BM and Rabbani M (2015) Prevalence and distribution of soil-borne zoonotic pathogens in Lahore district of Pakistan. Front. Microbiol. 6:917. doi: 10.3389/fmicb.2015.00917

A multidisciplinary, collaborative project was conducted to determine the prevalence and distribution of soil-borne zoonotic pathogens in Lahore district of Pakistan and ascertain its Public Health Significance. Using a grid-based sampling strategy, soil samples ($n = 145$) were collected from villages ($n = 29$, 5 samples/village) and examined for *Bacillus anthracis, Burkholderia mallei/pseudomallei, Coxiella burnetii, Francisella tularensis*, and *Yersinia pestis* using real time PCR assays. Chemical analysis of soil samples was also performed on these samples. The relationship between soil composition and absence or presence of the pathogen, and seven risk factors was evaluated. DNA of *B. anthracis* (CapB), *B. mallei/pseudomallei* (chromosomal gene), *C. burnetii* (IS1111, transposase gene), and *F. tularensis* (lipoprotein/outer membrane protein) was detected in 9.6, 1.4, 4.8, and 13.1% of soil samples, respectively. None of the samples were positive for protective antigen plasmid (PA) of *B. anthracis* and *Y. pestis* (plasminogen activating factor, pPla gene). The prevalence of *B. anthracis* (CapB) was found to be associated with organic matter, magnesium (Mg), copper (Cu), chromium (Cr), manganese (Mn), cobalt (Co), cadmium (Cd), sodium (Na), ferrous (Fe), calcium (Ca), and potassium (K). Phosphorous (P) was found to be associated with prevalence of *F. tularensis* while it were Mg, Co, Na, Fe, Ca, and K for *C. burnetii.* The odds of detecting DNA of *F. tularensis* were 2.7, 4.1, and 2.7 higher when soil sample sites were >1 km from animal markets, >500 m from vehicular traffic roads and animal density of <1000 animals, respectively. While the odds of detecting DNA of *C. burnetii* was 32, 11.8, and 5.9 higher when soil sample sites were >500 m from vehicular traffic roads, presence of ground cover and animal density of <1000 animals, respectively. In conclusion, the distribution pattern of the soil-borne pathogens in and around the areas of Lahore district puts both human and animal populations at a high risk of exposure. Further studies are needed to explore the genetic nature and molecular diversity of prevailing pathogens together with their seroconversion in animals and humans.

Keywords: ***Bacillus anthracis*, *Francisella tularensis*, *Coxiella burnetii*, *Burkholderia mallei/pseudomallei*, *Yersinia pestis*, soil chemistry, risk factors**

Introduction

Pathogens of public health significance such as *Burkholderia mallei/pseudomallei, Francisella tularensis, Yersinia pestis, Bacillus anthracis,* and *Coxiella burnetii* have significantly influenced human health throughout history (Sjöstedt, 2007; Ayyadurai et al., 2008; Oyston, 2008; Butler, 2009; Khan et al., 2013a) and anticipated to do so for the foreseeable future. These pathogens may enter the human body either through ingestion, inhalation or contact with contaminated soil and infected animals (Coenye and Vandamme, 2003; Ayyadurai et al., 2008; Oyston, 2008; Kersh et al., 2013; Khan et al., 2013a).

The incidence of *B. anthracis*, *F. tularensis*, *B. mallei/pseudomallei* and *Y. pestis* have been reported worldwide (Ayyadurai et al., 2008; Oyston, 2008; Butler, 2009). Prevalence of *B. anthracis* and *B. mallei* in humans and animals has been previously reported in Punjab province of Pakistan based on clinical findings and not supported by laboratory based confirmation. Further, little is known about the epidemiology of *F. tularensis* and *Y. pestis* in human and animal populations in Pakistan. Determination of the true prevalence of these pathogens is further complicated due to lack of a reporting system in the province of Punjab. The objectives of this study were to; (1) determine the prevalence of soil-borne zoonotic pathogens including *B. anthracis, B. mallei/pseudomallei, F. tularensis, Y. pestis* and *C. burnetii* in soil samples collected from villages in Lahore district, (2) identify soil components that favor or deter the presence of pathogens, and (3) identify risk factors associated with the presence or absence of the pathogen in the soil. It is anticipated that the findings of the study will provide information to undertake rigorous epidemiologic investigations focused on prevalence and distribution of soil-borne zoonotic pathogens in humans and domestic animals in Pakistan.

Materials and Methods

Study Area

The Lahore district in Punjab Province of Pakistan was selected for conducting the study. Lahore (31°15′–31°45′N and 74°01′–74°39′E) is bound in the north and west by the Sheikhupura district, Kasur district in the south and India in the east. The river Ravi flows on the north-western side of Lahore city (**Figure 1**). The study area is 217 m above sea level and covers a total land area of 1772 km^2. With a population exceeding 10 million, administratively the district is comprised of 10 towns, 271 union councils and 301 villages. Villages represent the lowest administrative unit, while a collection of villages represent a union council.

Sample Collection

Lahore district has 301 villages. Ten percent of these villages ($n = 29$) were randomly selected using Open Epi version 2.3.1

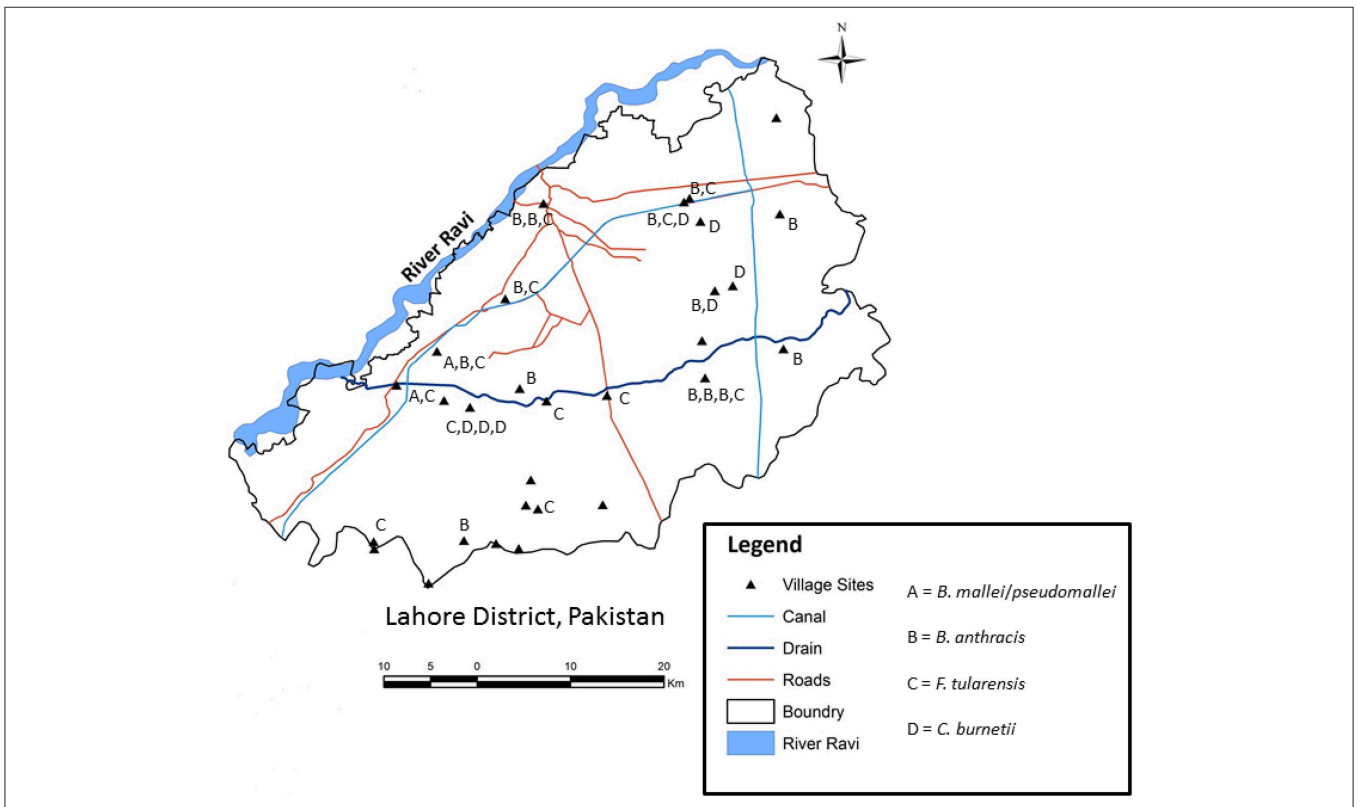

FIGURE 1 | Geospatial distribution of *Burkholderia mallei/pseudomallei*, *Bacillus anthracis, Francisella tularensis*, and *Coxiella burnetii* DNA in soil samples collected in Lahore district, Pakistan.

(http://www.openepi.com/Menu/OE_Menu.htm) software using a 5% confidence limit and 80% level of significance (**Figure 1**). From each village, five sites were identified and sampled by removing 3–5 inches of the top soil. Samples from four sites were locations that had both animals and humans dwelling in close proximity while the fifth site was located in an area where there was no evidence of apparent recent human and animal activity. Samples were collected using personal protective equipment (PPE). From each sample site, three separate samples were collected approximately within one square meter of the sample site. The three samples were pooled, mixed thoroughly and divided into three aliquots for soil chemistry (∼500 g), real time (RT) PCR assays for pathogens (∼200 g) and sample archives (∼500 g). Using a sample collection survey questionnaire, necessary information pertaining to risk factor analysis for a given sample collection site including GIS location, presence or absence of domestic animals, animal density, distance from animal market, main road and water source such as river, canal, streams, and drains (canal or channel system which carries sewage and rain water), presence or absence of vegetation and human dwelling was obtained.

DNA Extraction from Soil Samples

DNA from soil samples was extracted using PowerSoil® DNA Isolation Kit (MoBio, USA) as per the manufacturer's instructions. The quality ($A_{260/280}$ and $A_{260/230}$) of the DNA was assessed by spectrophotometry (NanoDrop, USA) while the quantity (ng/μL) was determined by Qubit fluorometer (Invitrogen, USA) using the DNA BR Assay kit (Invitrogen, USA) as per the manufacturer's instruction.

Real Time PCR Analysis

The prevalence of selected soil-borne pathogens was determined by RT PCR (CFX96, BioRad, USA). Previously reported oligonucleotides (primers and Taqman probes) were used for detection of *B. anthracis*, [plasmids containing capsular (CapB) and protective antigen (PA)], *Y. pestis* (Plasminogen activating factor, pPla gene), *F. tularensis* (Outer membrane) (Christensen et al., 2006), *B. mallei/pseudomallei* (Chromosomal gene) (this study) and *C. burnetii* (IS1111 gene) (Tozer et al., 2014) (**Table 1**).

RT PCR assay was optimized and validated using the positive controls (dsDNA PCR products) and proficiency testing samples provided by Drs. Francesconi and Patel Naval Medical Research Unit, Frederick, Maryland. RT PCR assays for detection of *B. anthracis*, *B. mallei/pseudomallei*, *F. tularensis* and *Y. pestis* were performed in 25 μL of reaction volume comprising of final concentration of 1X PCR buffer, 5 mM of $MgCl_2$, 0.25 mg/mL of bovine serum albumin, 0.25 mM of dNTPs, 0.6 μM of each forward and reverse primer, 0.025 μM of probe and 0.5 U of *Taq* polymerase along with DNA extracted from soil (10–30 ng). The cycling condition was as follows; one cycle of 95°C for 5 min followed by 45 cycles each of denaturation at 94°C for

TABLE 1 | List of PCR primers and probes used in this study.

Pathogen	Gene[a,b,c]		Sequence (5′–3′)
Bacillus anthracis	Capsular antigen (pXO2)[a]	FP	CAGATAATGCATCGCTTGCTTTAG
		RP	GGATGAGCATTCAACATACCACG
		Probe	CAGAGGCTCTTGGATTGATGAGGAAACAO
	Protective antigen (pXO1)[a]	FP	TTCAAGTTGTACTGGACCGATTCTC
		RP	TCCATCATTGTCACGGTCTGG
		Probe	CCGTAGGTCCAGCACTTGTACTTCGCTTO
Yersinia pestis	Plasminogen activating factor (pPla)[a]	FP	ATTGGACTTGCAGGCCAGTATC
		RP	ATAACGTGAGCCGGATGTCTTC
		Probe	AAATTCAGCGACTGGGTTCGGGCACA
Burkholderia mallei/pseudomallei	Chromosomal gene[b]	FP	GCAAATCACCTTCGATGCAAC
		RP	CTAATTGAACCGAACCTTCG
		Probe	TCATCGCGACACTGGAATCGATGCGACACAT
Francisella tularensis	Lipoprotein/outer membrane protein[a]	FP	CAGCATACAATAATAACCCACAAGG
		RP	TCAGCATACTTAGTAATTGGGAAGC
		Probe	TTACAATGGCAGGCTCCAGAAGGTT
Coxiella burnetii	IS1111 gene/Transposase[c]	FP	GTCTTAAGGTGGGCTGCGTG
		RP	CCCCGAATCTCATTGATCAGC
		Probe	AGCGAACCATTGGTATCGGACGTTTATGG

[a] *Primers (FP, forward primer; RP, reverse primer) and probes used in the study were as described by Christensen et al. (2006).*
[b] *this study.*
[c] *Primers and probes used in the study were as described by Tozer et al. (2014).*

5 s and annealing at 60°C for 20 s; and then one cycle of cooling at 40°C for 1 min. For detection of *C. burnetii,* the 25 μL of reaction volume contained 12.5 μL of qPCR mix (Thermo Scientific, USA), 10 pM of each primer and probe and 10–30 ng of template DNA. The cycling condition was as follows: 15 min incubation at 95°C followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Diethylpyrocarbonate (DEPC)-H_2O was used as a negative control in all the assays. DNA from the same extract underwent two independent RT PCR reaction for the studied pathogens. Soil samples that exhibited a positive RT PCR result were assayed a third time beginning from genome extraction and the PCR products (presence and size in bp) were assessed by agarose gel electrophoresis (3.0%) with the appropriate controls and a 50 bp ladder (Fermentas, Germany).

Soil Chemistry Analysis

The physical and chemical properties of soil samples were analyzed using validated techniques published previously for pH (McKeague, 1978), moisture (McLean, 1982), texture (Robert and Frederick, 1995), total soluble salts (Magistad et al., 1995), phosphorous (Frank et al., 2012), copper, chromium, nickel, manganese, cobalt, lead, cadmium, iron, sodium, potassium, calcium, and magnesium (Soltanpour and Schwab, 1977), nitrogen (Fierer et al., 2001) and organic matter (Nelson and Sommers, 1982).

Data Analysis

A total of 145 samples from 29 villages in Lahore district were examined for the presence of five pathogens. Results of the RT PCR analysis, soil chemistry and seven risk factors for each sample was compiled in a single Microsoft Excel spreadsheet. The presence or absence of a pathogen in relation to soil chemistry and seven potential risk factors was determined through student t distribution (*T*-test) and odd ratio (OR), respectively. Soil chemistry values (average count) were compared using the Tukey-Kramer (equal variance) or Dunnett's T3 (unequal variance) procedures. These two procedures were used due to unequal sample sizes observed in the two categories of a given value. The Tukey-Kramer procedure performs all pair-wise comparisons, testing whether the means are significantly different. The Dunnett's T3 procedure performs all comparisons with a control category. $P < 0.05$ was considered significant.

ORs were used to evaluate risk factors associated with pathogen. Observations to the seven risk factors were grouped by their categorical response (e.g., *yes*, *no*) to estimate if an observation had an influence on the presence or absence of the pathogen. For a potential risk factor, OR > 1 was considered to be associated with the outcome (presence or absence of pathogen) while it was considered vice versa for OR < 1. Confidence interval (CI, 95%) was used to estimate precision of the OR where a large CI value was considered a low level of precision and small CI was considered with higher precision. Statistical analyses related to soil chemistry were performed with SPSS (version17.0; SPSS Inc., Chicago, IL) and STATA (version 12.0, College Station, Texas, USA) while ORs were calculated through two by two frequency table available with OpenEpi version 2.3.1.

Results

Prevalence of DNA of Bacterial Pathogens in Soil Samples Collected in Lahore District

A total of 29 of 301 villages in Lahore district were examined for soil-borne pathogens including *B. anthracis*, *B. mallei/pseudomallei*, *C. burnetii*, *F. tularensis* and *Y. pestis*. None of the soil samples were positive for DNA of *Y. pestis*. Soil samples from 25 of 29 (86.2%) villages and 40 of 145 (27.6%) sample sites were positive for DNA of atleast one of the four pathogens. DNA of *B. anthracis* (CapB), *F. tularensis* and *C. burnetii* was detected in 11 (37.9%), 14 (48.3%) and 4 (13.8%) of 29 villages, respectively. None of the samples were positive for the DNA encoding for protective antigen (pOX1). *Burkholderia mallei/pseudomallei* was detected in two of 29 (6.9%) villages and two of 145 (1. 4%) soil samples. Both these villages (Medhipur and Nasir Gurj) shared the same major roadway and were adjacent to a water canal (**Table 2**).

Soil Chemistry

The pH of soil samples collected from different sites ranged from near neutral to alkaline (6.50–9.85). A total of 20 soil analytes were examined including moisture (0.50–42.24%), soluble salts

TABLE 2 | Soil sample sites positive for DNA of four soil-borne pathogens.

	Village (sample site)	*Bacillus anthracis*	*Burkholderia mallei/ pseudomallei*	*Coxiella burnetii*	*Francisella tularensis*
1	Bagrian			1	
2	Batapur	1			1
3	Bhaseen				4
4	Gawala Colony				1
5	Hadiara	1			
6	Halloki	1			
7	Halokeey				2
8	Hanjarwal			2	
9	Heir	3			1
10	Hira Singh	1			1
11	Jallo Pind	1			
12	Jiya Bagga				1
13	Kahna				1
14	Kot Bagh Ali			3	
15	Lakhodere				2
16	Mandhiyala	1			
17	Maraka				1
18	Medhipur	1	1		1
19	Nasir Gurj		1		
20	Paajiyan	1			
21	Pangali	1			
22	Raiwind Pind				1
23	Sanat Nagar	2			1
24	Sultankey				1
25	Tehra Pind			1	
	Total (%)	14 (9.6%)	2 (1.4%)	7 (4.8%)	19 (13.1%)

(0.20–50.21%), organic matter (0.05–7.25 mg/Kg), clay (0.0–15.0 mg/Kg), sand (72.0–99.0 mg/Kg), silt (0.00–115.0 mg/Kg), nitrogen (0.002–3.92 mg/Kg), phosphorus (1.00–146.67 mg/Kg), magnesium (0.12–82.24 mg/Kg), copper (0.026–3.08 mg/Kg), chromium (0.002–0.25 mg/Kg), nickel (0.002–0.38 mg/Kg), manganese (0.015–0.89 mg/Kg), cobalt (0.005–0.15 mg/Kg), lead (0.01–1.44 mg/Kg), cadmium (0.03–0.47 mg/Kg), sodium (0.06–150.78 mg/Kg), ferric (0.04–5.59 mg/Kg), calcium (0.09–103.52 mg/Kg), and potassium (0.12–194.84 mg/Kg). Among the analytes examined, the concentration of analytes including moisture, soluble salts, clay, sand, silt, phosphorus, magnesium, sodium, calcium, and potassium were particularly variable (**Table 3**).

The presence of *B. anthracis* (CapB) DNA was significantly associated with elevated levels of organic matter, chromium, cobalt and cadmium while it was significantly associated with low concentrations of magnesium, copper, manganese, sodium, ferrous, calcium and potassium. *F. tularensis* DNA was associated with low concentrations of phosphorous. Except cobalt, the presence of DNA for *C. burnetii* was associated with low concentration of magnesium, sodium, ferrous, calcium and potassium. None of the soil chemistry variables was found to be associated with presence of *B. mallei/pseudomallei* (**Table 4**).

Determination of Risk Factors Associated with Soil-borne Pathogens

Risk factors including presence of domestic animals, distance between the sampling site and animal market, main road and water source, presence of ground cover, animal density and the number of households in the village were evaluated to determine if any of these factors could be associated with the presence or absence of the pathogen at the sampling site (**Table 5**). It was observed that *B. anthracis* (CapB) and *F. tularensis* were identified in four villages located along the Lahore-Multan road (**Figure 1**). None of the risk factors were associated with *B. anthracis* (CapB) DNA in the soil sample. The presence of *F. tularensis* in the sample was positively associated with distance from the animal market [2.75 (1.02–7.40)], main road [4.14 (1.23–13.87)] and animal density [2.77 (1.04–7.40)], while for *C. burnetii* in soil sample, it was positively associated with the distance from the main road [32 (5.49–186.3)], vegetation [11.88 (2.17–64.86)] and animal density [5.91 (1.10–31.7)] (**Table 5**).

TABLE 3 | Concentration of soil analytes from villages in Lahore district.

Soil analyte	Mean	Range	Standard deviation	Standard error
pH	8.26	6.50–9.85	0.503	0.042
Moisture (%)	9.12	0.50–42.2	6.225	0.517
Soluble salts (%)	3.71	0.20–50.1	5.772	0.479
Organic matter	2.46	0.05–7.25	1.565	0.130
Clay (mg/Kg)	6.02	0.00–15.0	3.728	0.310
Sand (mg/Kg)	85.44	72.0–99.0	5.985	0.497
Slit (mg/Kg)	10.02	0.00–115	12.936	1.074
Nitrogen (mg/Kg)	0.18	0.002–3.92	0.371	0.031
Phosphorus (mg/Kg)	39.85	1.00–146.7	37.164	3.086
Magnesium (mg/Kg)	10.74	0.12–82.24	15.525	1.289
Copper (mg/Kg)	0.41	0.026–3.08	0.413	0.034
Chromium (mg/Kg)	0.13	0.002–0.25	0.060	0.005
Nickel (mg/Kg)	0.04	0.002–0.38	0.039	0.003
Manganese (mg/Kg)	0.07	0.015–0.89	0.127	0.011
Cobalt (mg/Kg)	0.07	0.005–0.15	0.046	0.004
Lead (mg/Kg)	0.44	0.01–1.44	0.224	0.019
Cadmium (mg/Kg)	0.22	0.03–0.47	0.112	0.009
Sodium (mg/Kg)	25.13	0.06–150.8	25.314	2.102
Ferric (mg/Kg)	0.59	0.04–5.59	0.904	0.075
Calcium (mg/Kg)	21.65	0.09–103.5	29.435	2.444
Potassium (mg/Kg)	34.80	0.12–194.8	51.334	4.263

Discussion

Presumptive diagnosis of a disease condition in humans or animals is largely based on clinical manifestations of the disease for a given geographic area and its population. The process of disease diagnosis is greatly enhanced when there is prior history of a similar disease condition, and this is greatly augmented with accurate clinical, laboratory and epidemiologic data. Therefore, timely identification of the etiologic agent using a validated and approved diagnostic test is critical to developing and implementing the most responsive disease prevention and control practices for a given geographic location and population.

Although in recent years, considerable efforts have been made to improve disease reporting, monitoring and surveillance in Pakistan; much needs to be done with respect to addressing emerging and re-emerging diseases. The Directorate of Animal Disease Reporting and Surveillance (ADRS) and Punjab Health Department in Punjab province of Pakistan periodically collect data on various disease conditions; however, the data collected is largely based upon clinical symptoms and/or with little laboratory diagnostic support. Further reporting for *B. anthracis* and *B. mallei* is exclusively done by ADRS.

In our study, real time PCR was the test of choice for the following reasons: (a) culture-based methods for highly dangerous pathogens such as *B. anthracis, B. mallei/pseudomaillei, C. burnetii, F. tularensis*, and *Y. pestis* requires a highly contained laboratory and trained personnel as these bio-threat pathogens could pose catastrophic human and animal threat in the event there is a lapse in chain of custody and biosafety practices, (b) the RT PCR assays used in the study are validated and are of very high sensitivity and specificity as compared to conventional PCR, and (c) the RT PCR assays allows simultaneous examination of several samples with high throughout and rapid turnaround time. The RT PCR assay and protocol used in our study is highly sensitive (97%) with detection limit as low as <100 genome copies in a given sample and can be used for a variety of matrices such as tissue, blood, soil and other environmental samples (Christensen et al., 2006). The RT-PCR assay for *C. burnetii* targeted a multicopy gene (IS1111 gene) that has much greater sensitivity than single copy gene targets (Tozer et al., 2014).

The use of manure, plowing during plantation season, or use of waste water and water from small canals used for irrigation

TABLE 4 | Soil properties and their association with the presence of soil-borne pathogens.

Soil component (mg/Kg)	Negative	Positive	*F*-test	*T*-test
	Avg. (Range)	Avg. (Range)		
	(*n* = 131)	(*n* = 14)		
Bacillus anthracis				
Organic matter	2.37 (0.0408–6.73)	3.29 (1.25–7.25)	0.357	0.035
Magnesium	11.75 (0.102–82.24)	1.20 (0.33–2.60)	0.000	0.000
Copper	0.42 (0.03–3.083)	0.27 (0.12–0.69)	0.048	0.014
Chromium	0.12 (0.002–0.25)	0.16 (0.12–0.23)	0.003	0.000
Manganese	0.07 (0.02–0.89)	0.029 (0.02–0.04)	0.045	0.000
Cobalt	0.06 (0.004–0.15)	0.092 (0.06–0.13)	0.000	0.001
Cadmium	0.21 (0.03–0.47)	0.27 (0.11–0.42)	0.030	0.024
Sodium	26.85 (0.06–150.70)	8.98 (0.38–15.30)	0.000	0.000
Ferrous	0.63 (0.04–5.59)	0.19 (0.09–0.33)	0.006	0.000
Calcium	23.79 (0.09–103.60)	1.61 (0.12–4.18)	0.000	0.000
Potassium	38.40 (0.12–194.80)	1.01 (0.07–4.70)	0.000	0.000
Francisella tularensis				
Soil component (mg/Kg)	**(*n* = 126)**	**(*n* = 19)**	***F*-test**	***T*-test**
Phosphorus	42.00 (1.00–146.70)	25.55 (5.0–107.20)	0.05	0.013
Coxiella burnetii				
Soil component (mg/Kg)	**(*n* = 138)**	**(*n* = 07)**	***F*-test**	***T*-test**
Magnesium	11.25 (0.12–82.20)	0.56 (0.27–0.68)	0.000	0.000
Cobalt	0.07 (0.005–0.15)	0.10 (0.08–0.14)	0.003	0.002
Sodium	25.05 (0.06–140.70)	7.04 (5.71–8.31)	0.001	0.000
Ferrous	0.61 (0.04–5.59)	0.19 (0.14–0.29)	0.049	0.000
Calcium	22.68 (0.08–103.60)	1.23 (0.98–1.56)	0.000	0.000
Potassium	36.48 (0.12–194.80)	1.53 (0.11–6.53)	0.000	0.000

of crops/fields could be attributed to prevalence of the soil-borne pathogens. Based on the observations of our study, it is difficult to derive a causal relationship between soil characteristics and the presence/absence of studied pathogens. However, the findings do provide some insights into the distribution of these pathogens in soils of the Lahore region. For instance, pH, organic matter, water activity (a_w, available water within microenvironment), availability of oxygen, CO_2/CO_3 and the presence of certain cations particularly calcium are considered important for the ecology, endemicity and virulence of *B. anthracis* in a given geographical area (Dragon and Rennie, 1995; Shen et al., 2002; Hugh-Jones and Blackburn, 2009; Koehler, 2009; Hammerstrom et al., 2011). While determining the historical distribution and molecular diversity of *B. anthracis* in Kazakhstan, Aikembayev et al. (2010) attributed higher frequency of disease outbreaks in southern and northern portions of the country to alkaline soil rich in organic matter than to central regions which are dominated by desert and where soil does not support the survival of spores. Although we found lower concentration of cations particularly calcium (1.61 mg/Kg or 1610 mEq/gram) at places where DNA to *B. anthracis* was detected than places where it was not detected, it was adequate to support sporulation and its survival in soil. For example, in an effort to determine a possible link between soil calcium and ecology of *B. anthracis*, Smith (personal communication 2003, http://www.oie.int/doc/ged/D7115.PDF, page 12) concluded that areas with calcium (>150 mEq/gram) and pH (>7) had an incidence of disease occurrence seven time more than the places lacking these parameters. Anthrax has been considered endemic in northern Punjab districts such as Chakwal and Jhelum in particular, where sporadic cases do occur during the rainy season. Epp et al. (2010) concluded that within high-risk regions, flooding in spring followed by hot and dry conditions, wet pastures, short grass length and high animal density could result in the persistence and subsequent occurrence of outbreak. Based on our study it can be inferred that soil with alkaline pH and increased organic contents could be suitable for persistence of *B. anthracis*. Virulent strains of *B. anthracis* contain plasmid pXO1 and pXO2 that encode toxins and capsule, respectively (Fouet and Mock, 1996). In our study, DNA of *B. anthracis* that encodes for capsular gene (pXO2, capsular antigen CapB) was identified, while the plasmid for protective antigen (pXO1) was not detected. This could explain the presence of non-virulent type of *B. anthracis* in soil samples from Lahore district and could perhaps explain the lack of any documented evidence of anthrax cases in humans and animals.

TABLE 5 | Risk factors associated with presence or absence of DNA of *Bacillus anthracis, Francisella tularensis,* and *Coxiella burnetii* in soil samples.

Criteria	*Bacillus anthracis*			*Francisella tularensis*			*Coxiella burnetii*		
	+ve	−ve	OR (95% CI)	+ve	−ve	OR (95% CI)	+ve	−ve	OR (95% CI)
DOMESTIC ANIMAL									
Present	10	106	0.58 (0.17–2.04)	14	102	0.65 (0.21–2.0)	5	111	0.60 (0.11–3.31)
Absent	4	25		5	24		2	27	
DISTANCE FROM ANIMAL MARKET									
>1 km	5	35	1.52 (0.47–4.86)	9	31	2.75 (1.02–7.40)	1	39	0.42 (0.04–3.63)
<1 km	9	96		10	95		6	99	
DISTANCE FROM MAIN ROAD									
>500 m	3	12	2.70 (0.66–11.05)	5	10	4.14 (1.23–13.87)	5	10	32 (5.49–186.3)
<500 m	11	119		14	116		2	128	
GROUND COVER									
Ground cover	4	25	0.58 (0.17–2.04)	5	24	0.65 (0.21–2.01)	5	24	11.88 (2.17–64.86)
No ground cover	10	106		14	102		2	114	
WATER SOURCE (CANAL/STREAM/DRAIN)									
<100 m	10	115	0.34 (0.09–1.24)	15	110	0.54 (0.16–1.85)	2	123	0.048 (0.00–0.27)
>100 m	4	16		4	16		5	15	
ANIMAL DENSITY									
<1000 animals	5	41	1.22 (0.38–3.86)	10	36	2.77 (1.04–7.40)	5	41	5.91 (1.10–31.73)
>1000 animals	9	90		9	90		2	97	
NO OF HOUSEHOLDS									
>300 houses/village	10	79	1.64 (0.49–5.53)	8	81	0.40 (0.15–1.07)	6	83	3.98 (0.46–3.93)
<300 houses/village	4	52		11	45		1	55	

The river Ravi which originates from the Himachal Pradesh, India serves as the northwest border of Lahore district (**Figure 1**). Through recorded history, this fertile river basin has nurtured and supported civilizations and, even in modern times, is fundamental to agriculture, livestock and human habitation. In our study, soil samples from 14 and 19 sample sites were positive for DNA of *B. anthracis* and *F. tularensis*, of which 6 and 7 sampling sites were adjacent to a road way or a canal. Further, both the villages where DNA of *B. mallei/pseudomallei* was identified were exclusively located on "Lahore-Multan road." This time traveled highway serves as major interstate road that joins the river Ravi at several places of its course. The said road is key to transport people, animals, agricultural and industrial products to other regions of Pakistan. Certain places and villages around the road serve as animal holding areas, auction markets, butcher shops and rest/shelter area for animals and their herders. Many of the positive sample sites were close to private and government-owned slaughter houses as well as animal markets along/around the road. Annually, several thousand animals pass/sheltered for a day or two along the road as well as villages around it. These animals are either sold alive in a nearby animal market or slaughtered for meat. It was also observed that these locations lacked designated areas for waste disposal and animal refuse for slaughter houses in particular. The waste is dispersed into adjacent fields and canals and is being used to irrigate the agriculture land in villages around this interstate road. It is therefore not surprising that we identified pathogens even away from places with more frequent human-animal activity. It was also noted that suburban housing developments and well established industries bordered this road. Based on the findings of the study, it can be inferred that the soil in this area is subject to considerable perturbations through animal, human and industrial activities. The high frequency of detection of all major pathogens in this region of Lahore district is of particular concern to public health and puts human and animal populations at a higher risk of exposure to these pathogens.

None of the soil samples showed the presence of DNA of *Y. pestis,* a vector-borne pathogen transmitted by rat fleas to humans. The long-term persistence of *Y. pestis* in soil and environmental factor contributing its survival is still yet to be fully understood. Perry and Fetherston (1997) reported that *Y. pestis* perishes quickly outside its host or vector, temperature exceeding 40°C and exposure to desiccation. Ayyadurai et al. (2008) showed that *Y. pestis* can be isolated from hydrated soils. In our study, with the exception of May–July, the temperature in the Lahore district is typically below 40°C and the soil remains hydrated through all seasons of the year. Historically, incidence of *Y. pestis* has been reported in coastal borders of sub-continent (India and adjoining areas). However, there has been no reported incidence of plague in the study area, which is consistent with the absence of *Y. pestis* soil DNA reported here.

F. tularensis was detected in soil samples from 14 of the 29 villages. As observed with Anthrax, there are no reported incidences or records of disease outbreaks or cases suggestive of tularemia in Lahore district. The incidence of

tularemia has been reported globally with varying relative virulence (Low/moderate/high) caused by the *Francisella* species or subspecies involved in the particular geography (Oyston, 2008). A number of cases of clinical infection and subsequent isolation as well as identification of *F. tularensis* has been reported from many countries of the Northern Hemisphere. Further, the strain isolated in North America has been found highly virulent as compared to the strains isolated from Europe or Asia (Sjöstedt, 2007; Oyston, 2008). Though it needs further molecular characterization at subspecies level in future, it is for the first time that DNA to *F. tularensis* has been detected in the environment from this part of the world and thus expanding its known range of occurrence worldwide. It has also been reported that *F. tularensis* may persist in the environment in a given geographical area without concomitant disease outbreaks (Sjöstedt, 2007). Several factors favor the persistence of *F. tularensis* in endemic areas and subsequent infection. These factors include climatic conditions, heat stress, limitation of potassium, cysteine/sulfur, CO_2 and iron that affects virulence and its survival in the soil (Olsufiev, 1966; Bernard et al., 1994; Deng et al., 2006; Sjöstedt, 2007; Lindgren et al., 2009; Alkhuder et al., 2010). Furthermore, *Francisella* spp. has been found to have affinity to low temperature, moisture, organic matter and hay/straw (Dennis et al., 2001). The role of potential reservoirs cannot be ignored where transmission of virulent strains (*F. tularensis* subspecies *tularensis*) is associated with rabbit, ticks and sheep, whereas, transmission of less virulent strain (*F. tularensis* subspecies *holarctica*) is associated with ponds, streams, lakes, river, and water associated species (Ulu-Kilic and Doganay, 2014). Interestingly, of the total soil sample examined, we found DNA of *F. tularensis* more at places <100 m of water canals/drains ($n = 15/19$, **Table 5**).

Burkhoderia mallei and *B. pseudomallei* are shown to represent a single genomic species by DNA-DNA hybridization, however each exhibit distinct biochemical properties, epidemiology and manifest different clinical symptoms in humans and animals (Rogul et al., 1970; Coenye and Vandamme, 2003). BLAST analysis was performed for the *B. mallei/pseudomallei* primers and probe used in our study and they were found to be equally applicable to the detection of chromosomal gene of both species. Two soil samples collected along the "Lahore-Multan" road were positive for this pathogen. We anticipated that the likelihood of isolating or identifying this organism was much higher in this region of Lahore compared to other sites as this area has many horses and mule stables and is well traveled for goods/material transport by them. *B. mallei* has been isolated from Pakistan (Hornstra et al., 2009), and asymptomatic horses and mules could transmit this organism without being detected (Hornstra et al., 2009; Khan et al., 2013b). *Burkhoderia mallei/pseudomallei* has been shown to survive in the soils and artificial environments involved in animal husbandry such as water troughs (Coenye and Vandamme, 2003; Hornstra et al., 2009) that were observed to be abundant along the roads and in communal stables within our sampling region.

To date, there have been no reported cases/outbreaks of Q-fever in humans and animals in Lahore district. Even in the absence of a reported outbreak, detection of *C. burnetii* in soil (Kersh et al., 2010) or dust and aerosols is not unusual (Schulz et al., 2005; Astobiza et al., 2011). The clinical signs of Q-fever mimic many other diseases with undifferentiated clinical symptoms including fever, nasal and chest congestion, myalgia, neuralgia, nasal and ocular discharges which, in the absence of a confirmed laboratory diagnosis, makes it very difficult to identify the disease (Tozer et al., 2014; Vanderburg et al., 2014).

In conclusion, the findings of our study demonstrate the presence of DNA of *B. anthracis*, *B. mallei/pseudomallei*, *C. burnetii*, and *F. tularensis* in soil samples collected from Lahore district of Punjab Province. Although we observed an association between the concentration of certain soil analytes and the presence of soil-borne pathogens, a more comprehensive study with a larger sample size will be required to fully examine this observation. Based on the collective experience of the investigators involved in this study, a unified human and animal active and passive surveillance program is key to understand the prevalence and distribution of the pathogens in humans and animals in Pakistan. The surveillance program must be complemented with a contemporary national and region laboratory network system to detect and identify zoonotic diseases of public health importance.

Author Contributions

Conceived and designed the experiment: BMJ, WRM, MZS, MR. Performed the experiment related work in Pakistan: MZS, AAA, TJ, AA, MAA, MABS, MB, AIM, MHC, AB, MN. Sampling and facilitation in relevant procedures: MZS, MAA, MR, KM, KP, SF, TY. Analyzed the data: BMJ, MZS, KP, TJ, AAA, MABS, MB, MHC. Wrote the manuscript: BMJ, MZS.

Acknowledgments

This work was supported by the Defense Threat Reduction Agency, Basic Research Award # HDTRA1-10-1-0080 to the Pennsylvania State University, USA (Prime Awardee) and University of Veterinary and Animal Sciences, Lahore Pakistan (Subcontract Awardee). The support of Livestock and Dairy Development Department, Punjab, the local veterinarians particularly Dr. Sarfaraz Chatha and farmers for facilitating soil sampling from selected villages of district Lahore is acknowledged. The authors acknowledge the support of laboratory technicians Muhammad Naeem Akhtar, Imran Shafique, Muhammad Arshad, Muhammad Asif, and Babar Nawab for their assistance in sample collection from the field.

References

Aikembayev, A. M., Lukhnova, L., Temiraliyeva, G., Meka-Mechenko, T., Pazylov, Y., Zakaryan, S., et al. (2010). Historical distribution and molecular diversity of *Bacillus anthracis*, Kazakhstan. *Emerg. Infect. Dis.* 16, 789–796. doi: 10.3201/eid1605.091427

Alkhuder, K., Meibom, K. L., Dubail, I., Dupuis, M., and Charbit, A. (2010). Identification of trkH, encoding a potassium uptake protein required for

Francisella tularensis systemic dissemination in mice. *PLoS ONE* 5:e8966. doi: 10.1371/journal.pone.0008966

Astobiza, I., Barandika, J. F., Ruiz-Fons, F., Hurtado, A., Povedano, I., Juste, R. A., et al. (2011). Four-year evaluation of the effect of vaccination against *Coxiella burnetii* on reduction of animal infection and environmental contamination in a naturally infected dairy sheep flock. *Appl. Environ. Microbiol.* 7, 7405–7407. doi: 10.1128/AEM.05530-11

Ayyadurai, S., Houhamdi, L., Lepidi, H., Nappez, C., Raoult, D., and Drancourt, M. (2008). Long-term persistence of virulent *Yersinia pestis* in soil. *Microbiology* 154, 2865–2871. doi: 10.1099/mic.0.2007/016154-0

Bernard, K., Tessier, S., Winstanley, J., Chang, D., and Borczyk, A. (1994). Early recognition of atypical *Francisella tularensis* strains lacking a cysteine requirement. *J. Clin. Microbiol.* 32, 551–553.

Butler, T. (2009). Plague into the 21st Century. *Clin. Infect. Dis.* 49, 736–742. doi: 10.1086/604718

Christensen, D. R., Hartman, L. J., Loveless, B. M., Frye, M. S., Shipley, M. A., Bridge, D. L., et al. (2006). Detection of biological threat agents by real-time PCR: comparison of assay performance on the R.A.P.I.D., the lightcycler, and the smart cycler platforms. *Clin. Chem.* 52, 141–145. doi: 10.1373/clinchem.2005.052522

Coenye, T., and Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ. Microbiol.* 5, 719–729. doi: 10.1046/j.1462-2920.2003.00471.x

Deng, K., Blick, R. J., Liu, W., and Hansen, E., J. (2006). Identification of *Francisella tularensis* genes affected by iron limitation. *Infect. Immun.* 74, 4224–4236. doi: 10.1128/IAI.01975-05

Dennis, D. T., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., et al. (2001). Tularemia as a biological weapon: medical and public health management. *JAMA* 285, 2763–2773. doi: 10.1001/jama.285.21.2763

Dragon, D. C., and Rennie, R. P. (1995). The ecology of anthrax spores: tough but not invincible. *Can. Vet. J.* 36, 295–301.

Epp, T., Waldner, C., and Argue, C. K. (2010). Case-control study investigating an anthrax outbreak in Saskatchewan, Canada–Summer 2006. *Can. Vet. J.* 51, 973–978.

Fierer, N. J., Schimel, R., and Zou, J. (2001). The influence of balsam poplar tannin fractions on carbon and nitrogen dynamics in Alaskan taiga floodplain soils. *J. Soil Biol. Biochem.* 33, 1827–1839. doi: 10.1016/S0038-0717(01)00111-0

Fouet, A., and Mock, M. (1996). Differential influence of the two *Bacillus anthracis* plasmids on regulation of virulence gene expression. *Infect. Immun.* 64, 4928–4932.

Frank, K., Beegle, D., and Denning, J. (2012). "Phosphorus," in *Recommended Chemical Soil Test Procedure for North Central Region.* North Central Regional Publication No 221(Revised). Available online at: http://extension.missouri.edu/explorepdf/specialb/sb1001.pdf (Accessed May 5, 2015).

Hammerstrom, T. G., Roh, J. H., Nikonowicz, E. P., and Koehler, T. M. (2011). *Bacillus anthracis* virulence regulator AtxA: oligomeric state, function and CO2-signalling. *Mol. Microbiol.* 82, 634–647. doi: 10.1111/j.1365-2958.2011.07843.x

Hornstra, H., Pearson, T., Georgia, S., Liguori, A., Dale, J., Price, E., et al. (2009). Molecular epidemiology of Glanders, Pakistan. *Emerg. Infect. Dis.* 15, 2036–2039. doi: 10.3201/eid1512.090738

Hugh-Jones, M., and Blackburn, J. (2009). The ecology of *Bacillus anthracis*. *Mol. Aspects Med.* 30, 356–367. doi: 10.1016/j.mam.2009.08.003

Kersh, G. J., Fitzpatrick, K. A., Self, J. S., Priestley, R. A., Kelly, A. J., Lash, R. R., et al. (2013). Presence and persistence of *Coxiella burnetii* in the environments of goat farms associated with a Q fever outbreak. *Appl. Environ. Microbiol.* 79, 1697–1703. doi: 10.1128/AEM.03472-12

Kersh, G. J., Wolfe, T. M., Fitzpatrick, K. A., Candee, A. J., Oliver, L. D., Patterson, N. E., et al. (2010). Presence of *Coxiella burnetii* DNA in the environment of the United States, 2006 to 2008. *Appl. Environ. Microbiol.* 76, 4469–4475. doi: 10.1128/AEM.00042-10

Khan, I., Ali, S., Gwida, M., Elschner, M. C., Ijaz, M., Anjum, A. A., et al. (2013a). Prevalence of *Burkholderia mallei* in equids of Remount Depot, Sargodha, Pakistan. *Pak. J. Zool.* 45, 1751–1756.

Khan, I., Wieler, L. H., Melzer, F., Elschner, M. C., Muhammad, G., Ali, S., et al. (2013b). Glanders in animals: a review on epidemiology, clinical presentation, diagnosis and countermeasures. *Transbound. Emerg. Dis.* 60, 204–221. doi: 10.1111/j.1865-1682.2012.01342.x

Koehler, T. M. (2009). *Bacillus anthracis* physiology and genetics. *Mol. Aspects Med.* 30, 386–396. doi: 10.1016/j.mam.2009.07.004

Lindgren, H., Honn, M., Golovlev, I., Kadzhaev, K., Conlan, W., and Sjöstedt, A. (2009). The 58-kilodalton major virulence factor of *Francisella tularensis* is required for efficient utilization of iron. *Infect. Immun.* 77, 4429–4436. doi: 10.1128/IAI.00702-09

Magistad, O. C., Reitemeier, R. F., and Wilcox, U. S. (1995). Determination of soluble salts in soils. *Soil Sci.* 59, 65–76. doi: 10.1097/00010694-194501000-00010

McKeague, J. A. (ed.). (1978). *Manual on Soil Sampling and Methods of Analysis*, *2nd Edn.* Ottawa, ON: Soil Research Institute, Agriculture Canada.

McLean, E. O. (1982). "Soil pH and lime requirement," in *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties, 2nd Edn.*, *Number 9 (Part 2) in Series*, ed A. L. Page (Madison, WI: American Society of Agronomy), 199–224.

Nelson, D. W., and Sommers, L. E. (1982). "Total carbon, organic carbon, and organic matter," in *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties, 2nd Edn.*, *Number 9 (Part 2) in Series*, ed A. L. Page (Madison, WI: American Society of Agronomy), 539–579.

Olsufiev, N. G. (1966). "Tularemia," in *Human Diseases with Natural Foci,* Vol. 2, ed Y. Pavlovsky (Moscow: Foreign Languages Publishing House), 219–281.

Oyston, C. F. (2008). *Francisella tularensis*: unravelling the secrets of an intracellular pathogen. *J. Med. Microbiol.* 57, 921–930. doi: 10.1099/jmm.0.2008/000653-0

Perry, R. D., and Fetherston, J. D. (1997). *Yersinia pestis* – etiologic agent of plague. *Clin. Microbiol. Rev.* 10, 35–66.

Robert, G. P., and Frederick, R. T. (1995). *Introductory Soil Science Laboratory Manual*. London: Oxford University Press.

Rogul, M., Brendle, J. J., Haapala, D. K., and Alexander, A. D. (1970). Nucleic acid similarities among *Pseudomonas pseudomallei*, *Pseudomonas multivorans*, and *Actinobacillus mallei*. *J. Bacteriol.* 101, 827–835.

Schulz, J., Rungem, M., Schroder, C., Ganter, M., and Hartung, J. (2005). Detection of *Coxiella burnetii* in the air of a sheep barn during shearing. *Dtsch. Tierarztl. Wochenschr.* 11, 470–472.

Shen, Y., Lee, Y. S., Soelaiman, S., Bergson, P., Lu, D., Chen, A., et al. (2002). Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylyl cyclase exotoxins. *EMBO J.* 21, 6721–6732. doi: 10.1093/emboj/cdf681

Sjöstedt, A. (2007). Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. *Ann. N.Y. Acad. Sci.* 1105, 1–29. doi: 10.1196/annals.1409.009

Soltanpour, P. N., and Schwab, A. P. (1977). A new soil tests for simultaneous extraction of macro and micronutrients in alkaline soils. *Soil Sci.* 8, 195–207.

Tozer, S. J., Lambert, S. B., Strong, C. L., Field, H. E., Sloots, T. P., and Nissen, M. D. (2014). Potential animal and environmental sources of Q fever infection for humans in Queensland. *Zoonoses Public Health* 61, 105–112. doi: 10.1111/zph.12051

Ulu-Kilic, A., and Doganay, M. (2014). An overview: tularemia and travel medicine. *Travel Med. Infect. Dis.* 12, 609–616. doi: 10.1016/j.tmaid.2014.10.007

Vanderburg, S., Rubach, M. P., Halliday, J. E. B., Cleaveland, S., Reddy, E. A., and Crump, J. A. (2014). Epidemiology of *Coxiella burnetii* infection in Africa: a one health systematic review. *PLoS Negl. Trop. Dis.* 8:e2787. doi: 10.1371/journal.pntd.0002787

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 04 July 2016
doi: 10.3389/fpubh.2016.00144

Toward the Development of a Sustainable Scientific Research Culture in Azerbaijan (2011–2015)

*Saida Aliyeva[1], Peter Flanagan[2], April Johnson[2] and Lisa Strelow[3]**

[1] Civilian Research Development Foundation, Baku, Azerbaijan, [2] Signature Science, LLC, Baku, Azerbaijan, [3] Bechtel National. Inc., Baku, Azerbaijan

This review especially describes the dangerous pathogens research program in Azerbaijan (AJ) funded by the US Defense Threat Reduction Agency under the Cooperative Biological Engagement Program (CBEP) from 2011 through 2015. The objectives of the CBEP are to prevent the proliferation of biological weapons; to consolidate and secure collections of dangerous pathogens in central repositories; to strengthen biosafety and biosecurity of laboratory facilities; and to improve partner nations' ability to detect, diagnose, report, and respond to outbreaks of disease caused by especially dangerous pathogens. One of the missions of the CBEP is therefore to increase the research skills and proficiency of partner country scientists. The program aims to fulfill this mission by sponsoring scientific research projects that exercise the modern diagnostic techniques available in the CBEP-engaged laboratories and the enhanced disease surveillance/control programs. To strengthen the local scientists' ability to develop research ideas, write grant proposals, and conduct research independently, in-country CBEP integrating contractor personnel have mentored scientists across AJ and conducted workshops to address technical gaps. As a result of CBEP engagement, seven research projects developed and led by AJ scientists have been funded, and five projects are currently in various stages of implementation. The Defense Threat Reduction Agency has also sponsored AJ scientist participation at international scientific conferences to introduce and integrate them into the global scientific community. The efforts summarized in this review represent the first steps in an ongoing process that will ultimately provide AJ scientists with the skills and resources to plan and implement research projects of local and regional relevance.

Keywords: defense threat reduction agency, Cooperative Biological Engagement Program, Azerbaijan, research, surveillance

OPEN ACCESS

Edited by:
Hillary H. Carter,
U.S. Department of State, USA

Reviewed by:
Paras Jain,
Albert Einstein College of Medicine, USA
Kavita M. Berger,
Gryphon Scientific LLC, USA

****Correspondence:***
Lisa Strelow
ls347@cornell.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *03 November 2015*
Accepted: *24 June 2016*
Published: *04 July 2016*

Citation:
Aliyeva S, Flanagan P, Johnson A and Strelow L (2016) Toward the Development of a Sustainable Scientific Research Culture in Azerbaijan (2011–2015). Front. Public Health 4:144. doi: 10.3389/fpubh.2016.00144

INTRODUCTION

The Defense Threat Reduction Agency (DTRA)'s Cooperative Biological Engagement Program (CBEP) is a US Department of Defense program that is part of the larger Cooperative Threat Reduction Program aimed at reducing chemical, biological, and nuclear threats. In former Soviet Union countries, the CBEP has demolished bioweapon production facilities, consolidated collections of dangerous pathogens, and supported peaceful research activities that employ knowledgeable personnel (1). The CBEP emphasizes scientific engagement as a means to improve biosafety, biosecurity, and disease surveillance capabilities through research, training, technology transfer,

infrastructure improvement, and sustainment activities with the goal of reducing the risks associated with infectious diseases and disease outbreaks caused by bioterrorism or endemic threats to public and animal health. As part of the Cooperative Threat Reduction Program, the CBEP works with Azerbaijan (AJ) and other partner countries to establish modern disease surveillance systems and a corps of scientists to study and develop countermeasures against endemic especially dangerous pathogens (EDPs).

The US Government, in cooperation with international partner institutes, has adopted a strategy that is designed to identify and promote CBEP research activities that are of mutual interest to the US Government and host countries. The goals of this partnership are to (1) support efforts to build biosurveillance and biosafety capabilities, (2) engage partner country scientists in hypothesis-driven research, (3) promote One Health initiatives, and (4) foster an international culture of responsible and ethical conduct in biological research. To meet these goals, the CBEP has upgraded laboratory facilities and implemented a comprehensive training program to ensure that AJ scientists are exposed to best practices and are equipped to carry out their disease surveillance responsibilities into the future. The CBEP has created and delivered training modules to enhance the diagnostic and epidemiological capabilities of AJ's scientific and technical staff and to promote biosafety and biosecurity (BS&S). The CBEP has also employed a training-of-trainers approach to foster long-term sustainability of the program.

The scientific community in AJ – composed of academic research institutes, universities, non-governmental organizations, and other international research funders (such as World Bank) – has a long history and is too complex to describe within the scope of this review. Therefore, only CBEP's role in AJ is discussed herein.

SCIENCE AND CBEP IN AZERBAIJAN

The CBEP was first implemented in AJ in 2007. Initially, the program provided funding to renovate or build 12 BSL-2 laboratories belonging to the Azerbaijan Ministry of Health, State Veterinary Control Service (SVCS) under the Ministry of Agriculture, and Ministry of Defense, so as to provide the physical infrastructure for the implementation of BSL-2 diagnostics and research (**Figure 1**). In 2011, Bechtel National, Inc. (BNI) became the CBEP integrating contractor in AJ. This review therefore describes the CBEP especially dangerous pathogens research program in AJ from 2011 through 2015 only.

At the national level, the Republican Anti-Plague Station (RAPS) and Republican Veterinary Laboratory (RVL) in Baku serve as the top-tier diagnostic institutions in AJ. The RAPS and RVL conduct confirmatory testing for EDPs and other diseases affecting humans and animals, respectively. Outside of Baku, the human threat agent detection and response system is represented by four regional, CBEP-enhanced Anti-Plague Division (APD) laboratories in Imishli, Khachmaz, Lankaran, and Shamkir. Similarly, the veterinary laboratory system outside of Baku is represented by the Zonal Veterinary Laboratories (ZVLs); the five CBEP-enhanced ZVLs are located in Barda, Gakh, Goygol, Guba, and Sabirabad. Each of the Baku and regional CBEP-enhanced laboratories is responsible for diagnosing EDPs and other infections in their home regions using modern molecular, immunological, and bacteriological methods. All of the CBEP facilities enter the laboratory test results for suspect EDP cases into the Electronic Integrated Disease Surveillance System (EIDSS), an electronic reporting system introduced by CBEP and implemented throughout the country.

Beyond the CBEP-renovated laboratories, AJ scientists from other facilities are also involved in CBEP research and training programs. These institutes include the Azerbaijan Veterinary Scientific Research Institute (AVSRI), which is responsible for veterinary biologic preparations and veterinary research; Azerbaijan State Agrarian University (ASAU), which offers veterinary education; Regional Veterinary Offices, which monitor animal health and report to the SVCS; and the Centers for Hygiene and Epidemiology, which monitor human diseases in the country and report to the MoH. Including these institutes in CBEP activities helps to encourage collaboration with the CBEP-engaged laboratories, with the goal of enhancing the national disease surveillance system.

In addition to laboratory renovations and research, CBEP administers an extensive training program to ministry-level and regional facility staff involved with EIDSS. In addition to EIDSS training, CBEP-training subjects include clinical recognition of infections caused by EDPs in humans and animals; epidemiology and veterinary epidemiology; BS&S; and PCR, bacteriology, and serology laboratory diagnostics. A description and evaluation of training conducted by CBEP in 2014 and 2015 was recently published by Johnson et al. (2).

CBEP RESEARCH IN AZERBAIJAN

Cooperative Biological Engagement Program research focuses on the specific pathogens listed in **Table 1**. Research activity in AJ has focused on EDPs of particular importance to AJ and the Trans-Caucasus region, including *Brucella*, *Bacillus anthracis, Yersinia pestis*, *Francisella tularensis*, avian influenza virus, and Newcastle disease virus.

Cooperative Biological Engagement Program funds two types of research projects: Cooperative Biological Research (CBR) and Threat Agent Detection and Response Activity Projects (TAPs). CBR projects are multi-year endeavors led by non-AJ collaborators with input and support from AJ scientists. To date, two CBR projects have been completed in AJ. The first CBR project (AJ-2) was a clinical, epidemiologic, and laboratory-based assessment of brucellosis that was conducted in collaboration with researchers from the United States Army Medical Research Institute for Infectious Diseases and Louisiana State University. The second CBR project (AJ-3) was aimed at mapping EDPs and was carried out in collaboration with researchers from the University of Florida.

Threat Agent Detection and Response Activity Projects are 1-year undertakings that may or may not include an outside collaborator and are smaller in scope than CBR projects. They are meant to be pilot projects to generate sufficient preliminary data to justify more extensive research projects. Since 2011, seven TAPs

FIGURE 1 | **CBEP-engaged laboratories in Azerbaijan**. Green circles represent the Zonal Veterinary Laboratories; red circles represent the anti-plague divisions; the orange circle represents the Republican level Laboratories including one laboratory each from MoH (Republican Anti-Plague Station), one SVCS (Republican Veterinary Laboratory), and one MoD (Center for Sanitary Epidemiological Control).

have been funded, two of which are complete (**Tables 2** and **3**). One of the completed projects focused on avian influenza and Newcastle disease viruses [TAP-9 (3)], while the other focused on viral and rickettsial pathogens in arthropod vectors in northern AJ (TAP-8). Five additional TAPs are in various stages of project implementation.

Initially, CBEP research in AJ centered on CBR projects led by international collaborators. The local scientists were passively involved in these projects – collaborators handled the bulk of the work, including developing research questions, writing proposals, analyzing results, and developing reports and manuscripts. As a result, AJ scientists were not developing the skills needed to sustain their research activities beyond the period of CBEP engagement. In order to increase the capacity of AJ scientists to conduct independent research, in 2011 BNI

proposed that CBEP-funded research going forward should center on projects developed by AJ scientists. DTRA approved this approach, and now CBEP is providing extensive mentorship, training, and support to improve the research skills and capabilities of AJ scientists through the implementation of TAPs.

Grant Writing Mentorship

The mentorship process begins when an AJ scientist submits a research idea to BNI for consideration. BNI works with the scientist to ensure that the research question is clear, achievable, and falls within the CBEP's scope. Once submitted to and approved by DTRA for further development, the AJ author develops a draft white paper in AJ, which BNI translates to English and reviews, providing feedback to the author that is aimed at improving the quality of the proposal. This phase is highly iterative; most white papers require several rounds of feedback before they are finalized. Telephone conversations and in-person meetings are held with project stakeholders as needed to clarify questions and discuss information to be included in the document. Once stakeholder consensus on the white paper is achieved, the paper is submitted to DTRA for approval; once approved, the draft–review–revise process described earlier is repeated as the AJ author develops a full proposal for DTRA's consideration. The author prepares a list of materials and supplies to include in the project budget, and a draft sampling schedule that accommodates project duration and participant availability.

Before submission to DTRA, all proposals are approved by the internal scientific review committee of the submitting AJ institution(s). This step helps to ensure that AJ science projects comply with all ministry and institutional procedures and requirements.

Defense Threat Reduction Agency reviewer feedback on submitted white papers and abstracts is verbally communicated pending translation of comments and recommended changes from English to Azerbaijani. Once complete, the AJ author receives a copy of the translated comments and BNI helps him or her revise the proposal to address reviewer concerns. Although time-consuming, this approach fully engages authors in the grant development process and provides AJ scientists with a deeper understanding of research proposal planning and development.

Since 2011, 32 project ideas have been submitted for consideration for CBEP funding (**Table 2**), of which eleven have been selected for white paper submission. Of those eleven, two have been completed: TAP-8 examined the prevalence of viral and rickettsial pathogens in arthropod vectors in northern AJ, and TAP-9 focused on detection of avian influenza and Newcastle disease viruses in the Barda region of AJ (**Table 3**). Both TAP-8

TABLE 1 | CBEP priority EDPs.

Human health	Animal health	Zoonoses
• *Yersinia pestis* (plague) • *Francisella tularensis* (tularemia) • Crimean-Congo hemorrhagic fever virus • Tick-borne encephalitis virus • Smallpox virus • *Clostridium botulinum*	• Capripox virus • Newcastle disease virus • African swine fever virus • Classical swine fever virus • Foot and mouth disease virus • *Burkholderia mallei* (glanders) • Rinderpest virus • Peste des petits ruminants virus	• Avian influenza virus • *Brucella* spp. • *Bacillus anthracis* (anthrax) • *Coxiella burnetii* (Q fever)

TABLE 2 | Number and progress of projects submitted by CBEP scientists (2011–2016).

AJ government entity	Total	Not Selected	In progress	Funded/ ongoing	Complete
Ministry of Health	12	9	0	2	1
State Veterinary Control Service	16	12	2	1	1
Joint MoH/SVCS	3	0	1	2	0
Ministry of Defense	1	0	1	0	0
Total	32	21	4	5	2

TABLE 3 | Proposal titles of completed, approved, and ongoing TAP projects (2011–2015).

Project	Title	Key words	Ministry	Status
TAP-8	Ecological and epidemiological study of viral and rickettsial pathogen prevalence in arthropod vectors in the northern part of Azerbaijan	Arboviruses, Azerbaijan, vector, arthropods, ticks, sandflies, mosquitoes, rickettsia, Q fever, CCHF, TBE, West Nile virus, PCR	MoH	Completed
TAP-9	Biosurveillance of avian influenza and Newcastle disease viruses in the Barda region of Azerbaijan using real time RT-PCR and hemagglutination inhibition	Avian influenza, Newcastle disease, Azerbaijan, environmental surveillance, live bird market, Hemagglutination inhibition, real-time RT PCR	SVCS	Completed
TAP-10	Ecological and epidemiological study of *Yersinia pestis* and *Francisella tularensis* in the northern part of Azerbaijan regions of Gusar and Khachmaz	*Francisella tularensis*, *Yersinia pestis*, ticks, fleas, Azerbaijan, PCR	MoH	Ongoing
TAP-11	Regional study of the ecology of anthrax foci in Georgia and Azerbaijan	Anthrax, Azerbaijan, Georgia, genotyping, GIS, bacteriology, PCR	SVCS and MoH	Ongoing
TAP-12	Isolation of *Brucella* species from the milk of lactating ruminants in the Goygol Rayon of Azerbaijan	Brucellosis, Azerbaijan, serology, ELISA, bacteriology, PCR	SVCS	Approved
TAP-13	Investigation of mosquito- and tick-borne arboviruses in Southeastern Azerbaijan	Arboviruses, Azerbaijan, vectors, ticks, mosquitoes, PCR, VectorMap	MoH	Ongoing
TAP-14	Detection and study of anthrax in central lowland region of Azerbaijan	Anthrax, burial sites, Azerbaijan, bacteriology, PCR	SVCS and MoH	Approved

and TAP-9 were developed and implemented by AJ scientists with support from CBEP. TAP-10, -11, and -13 are all being implemented (**Table 3**). TAP-12 and -14 are approved, but sampling has yet to begin.

Some of the proposals outlined in **Table 2** were not related to CBEP pathogens of interest and were therefore not selected by DTRA for further development. In these cases, BNI suggested other more suitable funding sources for these proposals, as applicable. Consequently, four non-EDP proposals were submitted to the Science Development Foundation under the President of the Republic of Azerbaijan for funding consideration. In some cases, the non-EDP projects received support from other organizations; for example, a rabies project was funded by the UK's Animal Plant Health Agency and described by Zeynalova et al. (4). These organizations provided funding for research, training programs, and workshops. Thus, different organizations provided additional funding for research, training programs, and workshops.

Research Mentorship

After a proposal is approved by DTRA, extensive mentorship is provided throughout project implementation. At the beginning of each project, a kick-off workshop is held to align expectations and familiarize project participants with the planned research. AJ scientists also develop data collection forms, which are reviewed by BNI for completeness and used to facilitate data capture. A BNI Science Coordinator (a local national staff member) is assigned to each project to guide participants, periodically accompany the team on field expeditions, and visit the laboratory to ensure that sample collection and diagnostic testing are being conducted safely and appropriately.

Each month, the AJ scientists provide updates on all activities and results for inclusion in a monthly research report to DTRA. This process acquaints project participants with the principles of report writing, data analysis, and effective communication on a routine basis, and prepares them to develop more extensive documents in the later phases of research. These monthly reports also help to identify issues that may require further attention from CBEP in a timely fashion. In addition, the monthly reports provide the raw material for comprehensive quarterly reports and final reports, which can ultimately serve as the basis for publications in international journals. For example, TAP-9, which was conducted by the RVL, was described in a publication by Zeynalova et al. (3). In addition, the final data for TAP-8, conducted by RAPS, are currently being analyzed for subsequent publication. Both projects were implemented by AJ participants with CBEP support during proposal development and research execution.

As with other former Soviet countries, scientific research was affected by the dissolution of the USSR that was indicated by a drop in scientific production after 1991. In 2006–2007, AJ, Kazakhstan, and Ukraine were publishing about half or less the number of scientific articles than they had in the early 1980s (http://www.science-metrix.com/) (5). National research funding for government health-care workers is limited, so international granting agencies are essential sources of support for infectious disease research. The CBEP has provided significant research opportunities to AJ scientists interested in investigating especially dangerous pathogens (outlined in **Table 1**).

Presentations for international conferences and manuscripts for submission to journals are also subjected to the draft–review–revise mentorship process. AJ scientists have presented project overviews and results of CBEP research at several international conferences/events, including the American Society of Tropical Medicine and Hygiene Annual Meeting, the Institute of Experimental and Clinical Veterinary Medicine, the Annual Meeting of the American Society for Virology, the International Research Conference on Brucellosis, and the Workshop on the Biology of Anthrax. The AJ project team members prepared the presentations and posters for these conferences with support from the BNI Science Team, as described earlier. Mentorship for conference attendance, abstract/poster development, and post-conference trip reports contributes to the scientists' abilities to meaningfully interact with the international scientific community.

One of the most important components of the CBEP is the enhancement of biosafety and security (BS&S) practices. Each research proposal is therefore accompanied by a protocol risk assessment tool. This tool captures the biorisks associated with a particular project and identifies mitigation activities to ensure that appropriate BS&S practices are employed in the field and in the laboratory. The assessment tool includes sections to describe the BS&S capabilities of the involved laboratories and other project facilities; the equipment and biosafety level to be used (and any required protocol/training/procedure enhancements to that biosafety level); biorisk management programs in place; project personnel and their training and experience levels; planned use of PPE; planned methods of decontamination and waste disposal; and pathogens to be investigated and methods of investigation. The principal investigator provides signature approval of the content of the tool before submission, as a mechanism to help reinforce BS&S compliance. Each project designates one individual to make sure that BS&S best practices are used during project implementation; this person works closely with the laboratory Biosafety Officer to reinforce BS&S compliance in the context of risks and mitigations associated with the research project. Research projects also incorporate research-specific BS&S training in advance of the project to reinforce the principles of safe research, and all CBEP-engaged laboratories receive annual BS&S refresher training regardless of involvement in research. Furthermore, the BNI Science Team provides continuous mentorship during research activities to address any BS&S gaps identified over the course of the project. If gaps are identified, additional training, mentoring, and other measures are taken to correct the deficiencies.

Grant Development and Scientific Writing Skills

In order to improve overall writing skills and grant/research proposal development skills, BNI coordinated a series of workshops conducted by the Civilian Research Development Foundation Global (CRDF). Topics included grant writing skills, scientific writing skills, and development of posters and abstracts for international conferences.

Two individuals from each CBEP-engaged laboratory were selected to participate in these workshops to ensure that each laboratory is equipped to effectively submit a proposal through

CBEP or a non-CBEP research funding source. The participant selection process incorporated feedback from AJ Laboratory Directors and the BNI Science Coordinators, who make regular visits to the laboratories and are familiar with laboratory staff. This effort was undertaken to identify the individuals who were most likely to benefit from the training and to be involved in research proposal development in the future. In addition to participants from the CBEP laboratories, scientists from other AJ organizations working with EDPs or collaborating with the CBEP laboratories in AJ were invited to attend the CRDF workshops. Representatives of local funding sources such as the Science Development Foundation under the President of the Republic of Azerbaijan and representatives from local offices of international funding sources were also invited to attend the workshops in order to introduce their programs and provide details on application requirements. The workshop materials were tailored to AJ with information on specific funding sources available to local scientists.

A total of six workshops were delivered by BNI and CRDF between 2011 and 2015 (**Table 4**). The first two workshops targeted scientists from ministry-level laboratories and institutes. Subsequent trainings were delivered to laboratory scientists from the regional CBEP-engaged laboratories and ASAU, which is located outside of Baku in Ganja. The most recent training, held in December 2015, focused on a small cadre of 12 research specialists selected by the ministries (6 from MoH and 6 from SVCS) to receive focused training on grant proposal development and submission. The expectation is that these 12 individuals will be able to mentor other AJ scientists on grant development and to assist with grant proposal submissions. A significant amount of workshop time was devoted to describe the proposal submission process for the Broad Agency Announcement (BAA) funding mechanism, through which AJ scientists will be able to apply for CBEP research funding once the integrating contractor is no longer in country.

Identification of Non-CBEP Funding Sources

Bechtel National, Inc.'s Research Coordinator routinely notifies AJ scientists of non-CBEP proposal funding opportunities in an effort to increase the probability that they will submit additional proposals on topics that may not be eligible for CBEP funding. The priorities and interests of the AJ scientists do not always align with CBEP priorities; this is reflected in the high number of research proposals received that focus on non-CBEP subjects. CBEP encourages scientists to pursue grants from non-CBEP sources to study these areas, since doing so provides valuable experience in proposal development and will open up additional opportunities for funding over time. CBEP offers mentorship to AJ scientists as they develop research questions and proposals, and translates information regarding non-CBEP funding opportunities.

As a result of CBEP funding notifications, AJ scientists independently applied for the "Mobility Grant" from the Science Development Foundation under the President of the Republic of Azerbaijan. During one CRDF workshop, CBEP informed workshop participants about the open calls for application to this foundation. Four proposals were subsequently submitted to the foundation, and were all selected for funding. This accomplishment demonstrates that AJ researchers are capable of developing successful proposals when made aware of funding opportunities.

DISCUSSION

As a result of increased mentorship and training offered through CBEP, AJ scientists are more involved in and capable of developing research ideas, writing grant proposals, and conducting independent research. Although the iterative translate–review–revise process is time-intensive, it represents an investment in the long-term development of critical skills that will enable AJ scientists to continue to develop competitive grant proposals long after CBEP engagement has ended. Overall, the scientists are invested in the projects and they feel more ownership when they, rather than collaborators, are leading the projects. This increased engagement has manifested in active development of abstracts for conferences, in seeking assistance to trouble shoot laboratory issues in real time, and in thinking ahead to where the data might take a project next. The skills they acquire as they

TABLE 4 | Grant writing workshops in Azerbaijan (2011–2015).

CRDF workshop topic	Location	Date	Organizations	Number of participants
Scientific writing and conference presentation skills	Baku	December 2012	RVL, SVCS, RAPS, RCH&E	22
Grant Writing	Baku	September 2013	RVL, SVCS, RAPS, RCH&E, AVSRI	35
Grant writing for regional CBEP laboratories representatives	Ganja	July 2014	ZVLs, APDs, ASAU	20
Advanced workshop on strategies for success in proposal development	Baku	December 2014	RVL, SVCS, ZVLs, RAPS, APDs, RCH&E, AVSRI, ASAU	43
Scientific writing	Baku	May 2015	RVL, SVCS, ZVLs, RAPS, APDs, RCH&E, AVSRI, ASAU	61
Professional skills training workshop strategies for success in proposal writing, making successful presentations and English language for grant writing	Baku	December 2015	RVL, SVCS, RAPS, Khachmaz APD, AVSRI	12
Strategies for success in proposal writing: Submitting to DTRA's Fundamental Research Broad Agency Announcement (BAA)	Baku	April 2016	RVL, SVCS, RAPS, Khachmaz APD, AVSRI	12

RAPS, Republican Anti-Plague Station; RVL, Republican Veterinary Laboratory; SVCS, State Veterinary Control Service; ZVL, Zonal Veterinary Laboratory; APD, Anti-Plague Division; RCH&E, Republican Centers for Hygiene & Epidemiology; ASAU, Azerbaijan State Agrarian University; AVSRI, Azerbaijan Veterinary Scientific Research Institute.

develop and implement research projects will be transferable to future research.

Addressing CBEP Research Goals

Cooperative Biological Engagement Program research priorities are being addressed in part through the implementation of the research program in AJ. These priorities include developing and enhancing sustainable partner country capabilities to (1) employ biorisk management best practices and principles, (2) conduct a modern and proactive disease surveillance mission, (3) comply with international reporting guidelines, and (4) promote and implement the One Health initiative (6).

Another goal of the research program is to foster a modern and proactive surveillance mission. Activities conducted during research projects, such as sample collection and diagnostic testing, are also relevant to surveillance. EDPs identified in the context of CBEP research projects must be reported via EIDSS. Research projects provide laboratory staff a chance to exercise these surveillance skills on a larger scale than they might otherwise encounter through routine diagnostic activities in their laboratories. In addition, the data collected can be used to establish baseline levels of knowledge on disease prevalence and distribution, and to characterize endemic agents; such information is critical for identifying outbreaks of disease and implementing control and prevention measures.

The last major goal of the program is to promote One Health initiatives. Significantly, some of the CBEP-sponsored research projects are collaborative projects between the MoH and SVCS. Such a novel approach in AJ is beneficial for encouraging communication and cooperation between agencies; during a zoonotic outbreak, ministries may be more likely to work together if they have a history of collaboration.

Challenges for CBEP Research Activity

Several obstacles to research execution have been identified during the implementation of the CBEP research program. Language barriers represent a significant hurdle. Most AJ CBEP scientists speak little English, which hinders their ability to search and reference international literature, and makes it more difficult to identify non-CBEP funding opportunities. The importance of English language skills has been emphasized during the grant writing workshops. Addressing this need falls out of the scope of CBEP, although the program is actively looking for English language training opportunities to share with the ministries.

Access to international literature is also limited; in part, this derives from not being subscribed to international scientific research journals for reason of cost, lack of access to electronic copies, and so on. This makes it difficult for the scientists who can read English literature to keep up to date on current research methods and techniques. AJ scientists rely heavily on AJ and Russian literature, which is often neither relevant nor comprehensive. The use of online translation software and applications has been incorporated into the grant writing workshops as a means of improving the scientists' ability to access funding announcements and other information available on the internet.

The competing professional obligations of personnel engaged in research projects present yet another challenge to successful research implementation. BNI has observed that dedicated and engaged scientists tend to be the most interested in research. These same individuals generally have more responsibilities, leaving less time for writing grants and conducting research. This problem exists throughout the system, where the top-performing staff tends to have the bulk of responsibilities. In order to address this challenge, BNI has encouraged regional scientists to take leadership roles in research projects and has encouraged all scientists participating in the grant writing workshops to submit project ideas.

Sustainability of Research Activities

The efforts summarized in this review represent the first steps in an ongoing process that will ultimately provide AJ scientists with the skills to plan and implement research projects of local and regional relevance. Having the skills and knowledge to develop research plans and apply for grants independently will allow AJ scientists to receive more funding for research, training, and other programs that might improve national capabilities. The long-term success of the research program will be highly dependent on Government of Azerbaijan involvement. Finally, the sustainability of research activity will depend on the ability of the MoH and SVCS to maintain laboratory equipment and infrastructure for conducting the proposed research. Research will need to be prioritized, with time allocated for scientists to work on proposal development, grant writing, and execution of research activities. Publications in peer-reviewed journal articles will increase the visibility of the AJ research program within the international scientific community, assist in developing collaborations, and increase the likelihood that scientists will receive funding in the future. In order to sustain research efforts in AJ, local investigators must be able to identify and successfully compete for international funding.

To increase the number of Azerbaijanis trained in grant writing, the final workshop event in early 2016 included a training-of-trainers component. Those who excelled in previous workshops and/or research projects, and who demonstrated an interest in training others were selected to attend this workshop; in all, 12 scientists participated in this phase of training, which began with a workshop conducted in December 2015. It is expected that these trainees will ultimately train other staff at their facilities and develop projects in collaboration with their colleagues.

Bechtel National, Inc. will continue to provide support and mentorship in project development and funding through the end of their period of performance. CBEP is transitioning to an online research proposal submission system using the US BAA process, which will provide a mechanism for AJ scientists to continue to apply for funding after BNI is no longer in the country. BNI mentored scientists on the new process by helping them set up accounts for each of the ministries; the next stage of the process will be to assist with submission of research projects through this new online portal. In addition, BNI's Research Coordinator will continue to share information on local and international funding sources with the CBEP scientists to encourage engagement outside of DTRA's purview. By working collaboratively, the challenges to develop a sustainable research program may be overcome for the mutual benefit of all of the participants engaged in this process.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

This research work was funded by Defense Threat Reduction Agency through the Collaborative Biological Engagement Program. The authors express their gratitude to Viktor Gasimov (Ministry of Health) and Siala Rustamova (State Veterinary Control Service under the Ministry of Agriculture) for continued program support. The authors are grateful to the AJ CBEP scientists engaged in the CBEP research program for their productive work. The authors are also grateful to Sonya Narodny for her assistance in reviewing the manuscript and the BNI Science Team for their involvement in mentorship and training. Finally, the authors acknowledge the contributions of CRDF Global for conducting the Grant Writing workshops.

REFERENCES

1. Young S, Willis HH, Moore M, Engstrom J. *Cooperative Biological Engagement Program (CBEP) Performance Capacities, Capabilities, and Sustainability Enablers for Biorisk Management and Biosurveillance*. Santa Monica, CA: RAND Corporation (2014). Available from: http://www.rand.org/content/dam/rand/pubs/research_reports/RR600/RR660/RAND_RR660.pdf
2. Johnson A, Akhundova G, Aliyeva S, Strelow L. Implementation and evaluation of a training program as part of the Cooperative Biological Engagement Program in Azerbaijan. *Front Public Health* (2015) 3:228. doi:10.3389/fpubh.2015.00228
3. Zeynalova S, Guliyev F, Vatani M, Abbasov B. Biosurveillance of avian influenza and Newcastle disease viruses in the Barda region of Azerbaijan using real time RT-PCR and hemagglutination inhibition. *Front Microbiol* (2015) 6:1128. doi:10.3389/fmicb.2015.01128
4. Zeynalova S, Shikhiyev M, Aliyeva T, Ismayilova R, Wise E, Abdullayev R, et al. Epidemiological characteristics of human and animal rabies in Azerbaijan. *Zoonoses Public Health* (2015) 62(2):111–8. doi:10.1111/zph.12119
5. Science-Metrix. *30 Years in Science [Online]*. (2012). Available from: http://www.science-metrix.com/30years-Paper.pdf
6. CBEP. *US Department of Defense, Defense Threat Reduction Agency, Cooperative Threat Reduction "The Cooperative Biological Engagement Program (CBEP) Research Strategic Plan: Addressing Biological Threat Reduction through Research*. (2015). Available from: http://www.dtra.mil/Portals/61/Documents/Missions/CBEP%20Research%20Strategy_FINAL_July%202015.pdf

Conflict of Interest Statement: The authors declare that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

REVIEW
published: 23 December 2015
doi: 10.3389/fpubh.2015.00270

Biothreat Reduction and Economic Development: The Case of Animal Husbandry in Central Asia

Robert Walker[1]* *and Jason Blackburn*[2]

[1] Department of Geography, Center for Latin American Studies, University of Florida, Gainesville, FL, USA, [2] Department of Geography, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA

Improving human welfare is a critical global concern, but not always easy to achieve. Complications in this regard have been faced by the states of the Former Soviet Union, where socialist-style economic institutions have disappeared, and the transition to a market economy has been slow in coming. Lack of capital, ethnic conflict, and political instability have at times undermined the institutional reform that would be necessary to enable economic efficiency and development. Nowhere are such challenges more pronounced than in the new nation states of central Asia, including Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan. Here, a severe climate limits agriculture, and industrialization has been inhibited by lack of infrastructure, low levels of human capital, and a scarcity of financial resources. These conditions are aggravated by the fact that the central Asian states are landlocked, far from centers of market demand and capital availability. Despite these daunting barriers, development potential does exist, and the goal of the paper is to consider central Asia's pastoral economy, with a focus on Kazakhstan, which stands poised to become a regional growth pole. The article pursues its goal as follows. It first addresses the biothreat situation to central Asian livestock herds, the most significant existing impediment to realizing the full market potential of the region's animal products. Next, it provides an outline of interventions that can reduce risk levels for key biothreats impacting central Asia, namely foot and mouth disease (FMD), which greatly impacts livestock and prohibits export, and Brucellosis, a bacterial zoonosis with high incidence in both humans and livestock in the region. Included is an important success story involving the FMD eradication programs in Brazil, which enabled an export boom in beef. After this comes a description of the epidemiological situation in Kazakhstan; here, the article considers the role of wildlife in acting as a possible disease reservoir, which presents a conservation issue for the Kazakhstani case. This is followed by a discussion of the role of science in threat reduction, particularly with respect to the potential offered by geospatial technologies to improve our epidemiological knowledge base. The article concludes with an assessment of the research that would be necessary to identify feasible pathways to develop the economic potential of central Asian livestock production as changes in policy are implemented and livestock health improves.

Keywords: central Asia, biothreats, economic development, conservation of natural resources, geospatial analysis

OPEN ACCESS

Edited by:
Nathan Wolfe,
Metabiota, USA

Reviewed by:
Sue Grady,
Michigan State University, USA
Craig Kiebler,
Metabiota, USA

***Correspondence:**
Robert Walker
roberttwalker@ufl.edu

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *24 May 2015*
Accepted: *12 November 2015*
Published: *23 December 2015*

Citation:
Walker R and Blackburn J (2015) Biothreat Reduction and Economic Development: The Case of Animal Husbandry in Central Asia. Front. Public Health 3:270. doi: 10.3389/fpubh.2015.00270

INTRODUCTION

Improving human welfare is a critical global concern, but not always easy to achieve. Complications in this regard have been faced by the states of the Former Soviet Union (FSU), where socialist-style economic institutions have disappeared, and the transition to a market economy has been slow in coming. Lack of capital, ethnic conflict, and political instability have at times undermined the institutional reforms that would be necessary for spurring economic efficiency and development. Nowhere are such challenges more pronounced than for the 64,000,000 people who reside in Central Asia, which includes the new nation states of Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan (1). Here, a severe climate limits agriculture, and industrialization has been inhibited by lack of infrastructure, low levels of human capital, and a scarcity of financial resources. These conditions are aggravated by the fact that the region is landlocked, far from centers of market demand and capital availability. Despite these daunting barriers, development potential does exist, and the goal of the paper is to consider Central Asia's pastoral economy, with a focus on Kazakhstan, whose ~18 million inhabitants are distributed over an expanse of 2,724,900 km^2, making it one of the world's largest countries.

Kazakhstan's economy has grown dramatically in recent years, lifting it to the status of a middle income country, with per-capita gross domestic product (GDP) at 11,550 US$. In that these gains are in large part based on the extraction of fossil fuels, the Kazakhstani government seeks to diversify its economic development strategy with an eye toward expanding agricultural exports, particularly beef (2). This reflects genuine opportunity, given global market expansion due to rising incomes and changing consumption patterns (3). Increasingly, middle income countries are finding ways to engage in the international trade of beef, despite continuing dominance by large producers, such as the US and Brazil (3). Extensive natural rangelands, together with a long-standing cultural tradition of animal husbandry, create a significant potential for Kazakhstan based on livestock management. That said, a number of issues constrain this potential at the present time, and it is the goal of the present paper to address one of them, namely, the regional biothreat situation.

The article pursues its goal as follows. It starts by describing the main biothreats presently affecting central Asian livestock herds, namely, foot and mouth disease (FMD) and Brucellosis. Like its central Asian neighbors, Kazakhstan's cattle herds and small stock consisting of goats and sheep have faced periodic problems with FMD and Brucellosis, and both diseases remain under official government surveillance (4). After this, the article moves on to a discussion of policy interventions that have managed to control or eradicate FMD, whose outbreaks bring substantial economic losses to those engaged in international trade. Policy is considered in the context of a case study of Brazil, a country that has largely suppressed FMD and, as a consequence, emerged as a major beef exporter. Parallels with Kazakhstan make the Brazilian experience relevant to Kazakhstani development efforts. After addressing policy, the article considers the role of computational science in threat reduction through the analysis and prediction of outbreak patterns. The article concludes with an assessment of the research that would be necessary to identify feasible pathways to develop the economic potential of Kazakhstani livestock production.

THE BIOTHREAT SITUATION IN CENTRAL ASIA

Livestock herds in many parts of the world are vulnerable to disease agents, some of which are capable of infecting humans. Two of the most significant with respect to economic impacts are FMD, a picornavirus of the genus *Apthovirus*, and Brucellosis, a bacterial zoonosis of the genus *Brucella*. FMD affect bovids and other hooved animals, both domestic and wild, causing fever and raising blisters (vesicules). Susceptible animals include cattle, pigs, sheep, goats, and camels. Although young cattle often die, mortality is low for mature animals, and impacts come from reduced outputs of animal-based products, such as milk and meat. Cattle and sheep are also susceptible to Brucellosis, a bacterial infection that can pass to the human population, where it is known variously as Malta Fever, Undulant Fever, etc. Human exposure typically occurs through the consumption of unpasteurized milk, and causes febrile symptoms, muscular pain, and sweating. Antibiotics have eliminated human mortality, which was never very high, although chronic sequelae (e.g., sacroiliitis, hepatic disease, endocarditis, and meningitis) can be serious. As for animal populations, *Brucella* causes economic damages by inducing spontaneous abortions, known in extreme cases as *abortion storms*. This obviously lowers reproductive potential and therefore rates of herd expansion, with commercial consequences. Effective vaccines have been produced for FMD (5), but efficacy is difficult to assess, and for Kazakhstan estimates do not exist. Moreover, the virus (FMDV) is capable of changing and possesses multiple serotypes, as discussed below. Thus, FMD prevention programs based on vaccination must remain vigilant to an evolving pathogen. As for Brucellosis in animals, a vaccine exists but prevention is best achieved by pasteurization of milk and cheese.

Epidemiology of FMD and Brucellosis in Kazakhstan Today

We now consider the epidemiology of FMD and Brucellosis, as well as challenges to formulating eradication and control policy given Kazakhstan's ecological context. This is followed by an overview of recent Kazakhstani history, which has significant implications for veterinary policy and practice.

Foot and Mouth Disease

Foot and mouth disease is caused by a positive, single-stranded RNA virus, which possesses seven known serotypes (O, A, Asia 1, C, SAT 1, SAT 2, and SAT 3), although serotype O is the most prevalent (6). The disease spreads easily, as infected animals shed the virus in secretions and excretions; transmission can be airborne, through contact with animal fluids, or via mechanical transmission of infected materials between properties (7). Overland airborne transmission can exceed 10 km, which makes FMD particularly difficult to contain (8). ELISA tests are capable of detecting the presence of antibodies to FMDV (test prevalence),

and also of identifying false positives due to prior vaccination (4). Such procedures provide imperfect measures of FMD (and Brucellosis) infection, although test prevalence may be used to estimate actual prevalence via Bayesian techniques (9). Despite diagnostic limitations, the Kazak National Reference Veterinary Center (NRVC) has reported serotypes O, A, and the A22 subtype in Kazakhstan, with evidence of disease in cattle, small stock (sheep and goats), swine, and camels (10). Between 1955 and 2007, (test) seroprevalence of FMD in cattle was assessed nationally at 3.78% for type A, 4.3% for type O, and 2.72% for A22. For small stock, the respective rates were 8.86, 9.63, and 3.83%, and for swine, 6.77, 6.32, and 7.54. Only types A and O were reported in camels, at 0.96 and 7.11%, respectively (10). Geographically, virus type O shows wide distribution, with cattle and small-stock infections reported in nearly all Kazakh oblasts (state/province equivalents). A similarly wide range is documented for serotype A, with small-stock reports primarily concentrated in southern and north central oblasts. A post-soviet survey of nearly 1,000 animals in 1997–1998 (six oblasts) showed high FMD rates in cattle that ranged from 2.9 to 52.8%, with the highest rate found in southern Kazakhstan oblasts (4). Rates were lower in small stock in that survey (0–22.0%), with the highest prevalence in the Aktiubinsk oblast. Outbreaks of types A and O have been reported in 2011 and 2012. FMD appears to occur in all oblasts, with the greatest risk in southern and eastern Kazakhstan, along the Kyrgyz, China, and Russian borders, illustrating a significant trans-boundary risk of transmission (11, 12). **Figure 1** illustrates the zones of risk according to NRVC in 2013, as defined by the concentration of cases.

Brucellosis

The newly formed Central Asian Republics represent one of the most significant loci of human Brucellosis infections in the world (13). With respect to Kazakhstan, the human incidence rose from 4.7 in 1967 to 15.3 cases per 100,000 in 1986, a direct consequence of FSU policy encouraging meat and dairy production (14). Rates remained high following the FSU's collapse after 1991, with an incidence lingering at 11.6 per 100,000, in line with the Lundervold et al. (4) estimate of 10.8 per 100,000 in 1989. Grushina et al. (15) reported annual morbidity at 17.5 per 100,000, noting that the Almaty region exceeds the national rate with 22.9 cases per 100,000 in 2006. Almaty's sub-districts Enbeksi-Kazakh and Zhambyl show very high rates, at 56.4 and 32.1, respectively (15). Pappas et al. (13) report that human *Brucella* infections in both Kazakhstan and Tajikistan are on the rise, on the basis of an evaluation of OIE and WHO reports. As with FMD, ELISA tests detect antibodies to *Brucella* in animals, and a serological survey (1997–1998) documented Brucellosis in cattle and small stock across eleven rayons (county/district equivalent) in south central Kazakhstan (4). Government statistics for small stock indicate seroprevalence (total sero-positive divided by the sample population) ranging from 0 to 1.1%, as compared to survey-specific rates of from 0 to

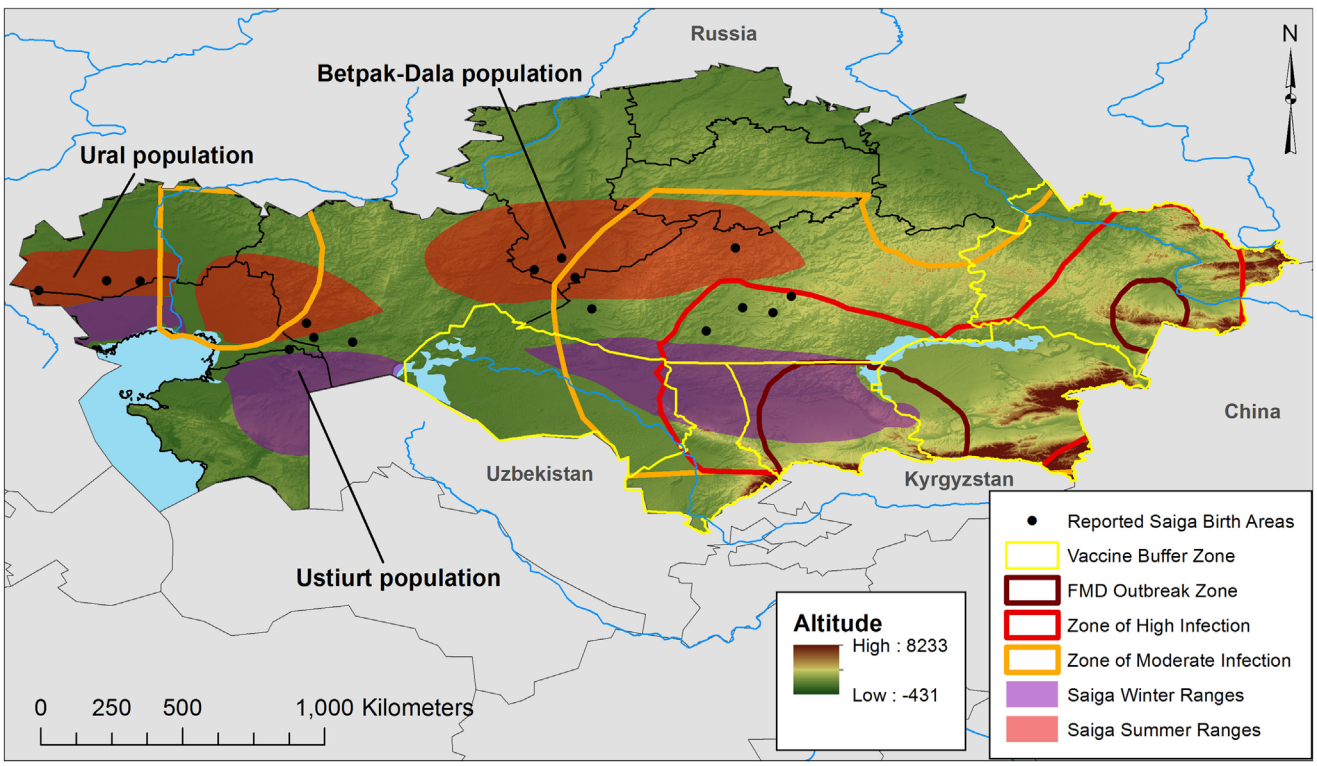

FIGURE 1 | The wildlife factor. Map of Kazakhstan illustrating the recent FMD outbreak and risk zones and the current vaccination buffer, as defined by Sytnik et al. (11) after the 2011–2012 livestock FMD outbreaks. *Saiga* populations, birthing areas, and seasonal ranges were adapted from Bekenov et al. (56) and Fry (57) to illustrate areas where surveillance and disease control should consider wildlife populations and conservation issues.

1.8% (4). Infection in neighboring countries may reach even higher levels, indicating a worrisome region-wide disease background. Seroprevalance in Tajikistan ranges from 0.53 to 6.96% at the rayon level (16), and clusters of sero-positivity in Armenian cattle and small stock show strong variation across time and space (17).

The Wildlife Factor

Kazakhstani rangelands cover 1.8 million km^2, about 70% of the national territory, and a semi-arid climate (300 mm annual precipitation) produces forage capable of supporting large populations of ruminant herbivores, both domesticated and wild (18). It should come as no surprise then that serology has documented Brucellosis in both livestock and multiple taxa of Kazakh wildlife, including maral deer, mountain sheep, mountain goats, roe deer, and *Saiga* antelope (14). There is also historical evidence of FMD between 1955 and 1974. Outbreak control for either disease is especially complicated by the presence of large herds of *Saiga* antelope that group into three major populations – the Ural, Ustiurt, and Betpak-Dala – spread across thousands of kilometers of longitude. This east–west distribution is complemented by north–south migrations between winter and summer grazing lands. Given its range and mobility, the *Saiga* antelope could carry both pathogens over large parts of Kazakhstan, where it might easily spread. This is suggested by **Figure 1**, which illustrates the risk zones for infected livestock during recent outbreaks (2011–2012). As shown, the zones of moderate infection extend deep into Kazakhstan, in a manner suggesting that seasonal antelope movements play a role in FMD livestock outbreaks; likewise, infected livestock could spread the disease to Saiga, which raises conservation issues, given the antelope is recognized by the Convention on International Trade in Endangered Species (CITES). Further research is necessary to document the exact mechanism and extent of cross-species transmission.

Recent Historical Considerations

The biothreat situation in Kazakhstan today results from an interaction between environmental conditions and a turbulent history of absorption into the USSR, the trauma of a devastating famine that killed nearly 1.5 million people, and ethnic tensions resulting from the Soviet Union's policy of distributing Russian nationals across the far reaches of its socialist empire (19). Prior to its incorporation into the USSR in 1930, the Kazaks practiced a mainly small-stock nomadism that accessed distinct ecological zones with two annual cycles. These comprise a latitudinal movement starting in the spring and covering 200–2000 km along a south–north axis, and an altitudinal one from the plains to the mountains during summer months (18). Sovietization refocused the Kazakhstani rural economy on cattle and wheat, and imported new agricultural institutions in the form of large state enterprises. Investments in veterinarian services were made, and agricultural activity in general became more capital intensive. That said, the state enterprises and the political nature of the USSR disrupted traditional forms of governance based on family clans (20).

Economic and social adjustments following the collapse of the USSR proved difficult, as was the case throughout Central Asia, with the physical constraints of its climate and geography. The Kazaks resumed old nomadic practices, but without the benefit of the social structures and institutions that had regulated transhumance and resource access for centuries (20, 21). It should come as no surprise that the early years of transition to a market economy brought widespread economic dislocation. The small-stock herd fell from 34.2 to 13.7 million animals between 1993 and 1996 (4). Similarly, the cattle herd of 7 million animals in 1995 fell to 4 million by 2000, with a slow recovery to 6 million animals by 2010 (22). From 1995 to 2010, the share of beef cattle in the national herd fell from 4 to 1%, and slaughter weights declined to 299 kg, which compares, for example, to 440 kg in Argentina (22). These dramatic and difficult transitions have dislocated herds, particularly with sell offs from large state enterprises to individuals and families. One consequence is a general lack of awareness about the immune status of animals on part of livestock owners and veterinary surgeons (4).

The disease agents as described present challenges to Kazakhstani development strategies targeting beef, given that the Sanitary and Phytosanitary (SPS) guidelines promulgated by the Office International des Epizooties (OIE; now the World Organization for Animal Health), which at the present time would probably disallow export to the markets within its purview. Moreover, export itself brings risk, given outbreaks of FMD and Brucellosis can prove costly. The 2001 FMD outbreak in the UK provides a case in point. The epidemic, which began on a pig farm in February, 2001, spread to at least 40 other properties in just a few weeks. By the end of September, after which no more outbreaks occurred, FMD had been reported on 2026 properties. Culling took place on these, neighboring, and proximity properties, totaling to ~8131. Altogether 4 million animals were slaughtered to control the outbreak, with an additional 2.5 million on "welfare grounds," numbers large enough to obscure any estimate of prevalence (23–26). By the time, the UK was declared FMD-free and cleared for export in January 2002, the disease had cost ~9 billion $US for culling, emergency vaccinations, and intensified surveillance (3, 27). Losses due to trade, pursuant to OIE and World Trade Organization (WTO) regulations, also added to the ledger. The UK FMD outbreak provides a hard lesson for all countries seeking to build export earnings through the trade of animal products.

Although a land-locked nation, Kazakhstan is surrounded by large markets in China (population 1.4 billion) and Russia (population 143 million) where climatic conditions significantly constrain animal husbandry. Adding to this and still within reach are the desert countries of the Middle East, and the densely settled lands of the European Union. Kazakhstan's Ministry of Agriculture is currently aiming to increase exports by orders of magnitude in no more than 5 years, from today's 1,000 metric tons to 180,000 by 2020 (2). Pursuant to this development objective, Kazakhstan has initiated herd improvements with the purchase of Angus and Hereford cattle from the US, Canada, and Australia, in an effort to raise the genetic qualities of its resident animals (2). But translating genetic quality into a robust export sector will require a number of additional investments, as, for example, in sanitary infrastructure capable of maintaining OIE standards for international trade in food products. As a member of both the Customs Union of the former Soviet Block, and the WTO, Kazakhstan has committed itself to such improvements with

sizeable budget allocations (28). Although some importers are willing to forgo OIE's stamp of approval, it is within Kazakhstan's long-run interests to build healthy herds of cattle and small stock.

THE SOUTH AMERICAN EXPERIENCE

Improved animal sanitation has proven economically important throughout the world, and contributed substantially to the development of countries with comparative advantage in land resources. This is especially true for South America, where the agricultural sector has often functioned as an engine of growth. With respect to livestock and meat products, Brazil and Argentina come immediately to mind as countries that improved their economic well-being by promoting international trade among their producers. Although Argentina has deemphasized livestock herding in favor of field crops like soybeans over the past decade, ranching long generated considerable export earnings (29). For its part, Brazil has joined the world stage as a powerful BRIC country, despite its current economic difficulties. This transformation has been partly enabled by agriculture, which includes management of the world's largest cattle herd, and position number two as a global exporter of beef (30). Brazil's ascension as a globally significant beef exporter has taken about a decade, given no Brazilian state was declared FMD-free until 1998.

Thus, South American countries potentially provide lessons in the leveraging of economic development outcomes from strategic interventions by federal governments in animal husbandry, and by engagement in export markets more generally. This is accentuated for Kazakhstan by similarities along physical, social, and economic dimensions, particularly with respect to Brazil. Both Brazil and Kazakhstan are large countries, possessing abundant land resources, with Brazil covering 8,515,767 km^2. Low levels of population density and the persistence of natural environments throughout South America means that Brazil retains within its boundaries ecological reservoirs of the FMD and Brucellosis disease organisms, as is the case in Kazakhstan (31, 32). As for levels of economic development, Brazil and Kazakhstan are also similar, with per-capita GDP reaching 11,690 US$ in Brazil, comparable to that of Kazakhstan (see above). We now consider efforts to control and eradicate FMD in Brazil, which spanned much of the twentieth century and involved a long-term process of policy adaptation. Although not discussed explicitly, the lessons learned in controlling FMD apply to Brucellosis and other biothreats.

Combatting FMD in Brazil

The relocation diffusion of FMD from Europe to South America occurred in the late nineteenth century, infecting herds in Brazil, Argentina, and Uruguay (33). Nevertheless, Brazil did not grow serious about solving the problem until the founding, in 1951, of the Pan-American Center of FMD (PANAFTOSA) in Rio de Janeiro. An initial strategy focused on prevention of the disease altogether, as Brazil (with help from the Word Health Organization) developed an FMD vaccine and provided credit lines to ranchers for implementing sanitary procedures (34). Despite these various initiatives, continuous outbreaks of FMD eroded both South American and Brazilian dreams of a continental export economy based on beef. An awareness that its FMD policies were not working, together with a growing interest in export on part of the private sector, inspired a significant policy shift, not only in Brazil but also throughout the continent. Consequently, the countries of South America agreed to the 1987 Hemispheric Plan for the Eradication of FMD, or PNEHA (35). This plan pursued a vigorous vaccination campaign of continental proportions, and declared a 95% coverage for the South American herd by 1995. The veterinary strategy of PNEHA addressed the disease epidemiology of cattle systems, with a focus on FMD-endemic areas and on the spatial links between grazing ranges and fattening operations (33). PNEHA responded immediately to outbreaks by controlling animal movements between affected and unaffected areas, and by mass vaccinations of susceptible animals; in areas disease-free before an outbreak, PNEHA recommended culling of affected and exposed animals (36).

Brazil's implementation of PNEHA via its Ministry of Agriculture in 1992 soon began to bear fruit, with a multipronged approach involving vaccination campaigns, capacity building of dedicated bureaucracies (e.g., Department of Animal Health), and the control of animal movements. The OIE had by then set international sanitary standards for the trade of animal products, creating strong incentives for exporters to improve livestock health. In addition and fortuitously for Brazil, OIE relaxed its requirement that countries be entirely free of FMD before engaging in trade. Starting in 1992, beef and beef products could originate from areas within a country certified to be FMD-free, even if the disease existed elsewhere inside national borders. The Brazilian approach aimed to eradicate FMD by regionalizing and decentralizing its efforts, and by stimulating the formation of public and private partnerships (37).

The regionalization, keyed to OIE's new spatial sensitivity to the environmental circumstances of animal husbandry, partitioned Brazil into five territorial extents, or *circuits*, to be managed individually in the fight against FMD, and which could then source export goods once FMD had been internally controlled (38). PNEFA also promoted decentralization by transferring programmatic responsibilities to Brazil's 26 states, and by direct involvement of civil society through outreach to producer associations. Ultimately, PNEHA implemented an effective division of responsibilities across both civil society and government (37). At the federal level, the Ministry of Agriculture acted as overall program manager and credit source, while individual states provided front-line veterinary services. For its part, the private sector managed culling during outbreaks, and created an emergency fund for vaccination and for the financial strain of monetary losses (37).

Figure 2 depicts PNEFA's dramatic impact on the FMD status of the Brazilian herd. The reported number of outbreaks is 589 in 1995, after which it drops precipitously, falling by an order of magnitude through the late 1990s. By 2002, outbreaks reach 0, with an uptick through 2006, after which the number returns to 0. The decisive drop appears to occur in the 1990s, in correspondence with Brazil's policy shift (39). FMD control manifests a distinct spatial pattern for the Brazilian case, with a move from southern to northern latitudes. Before 2000, only two southern states (Rio Grande do Sul and Santa Catarina) enjoyed OIE certification, but by 2004 the situation had changed dramatically, with most of southern and central Brazil engaged

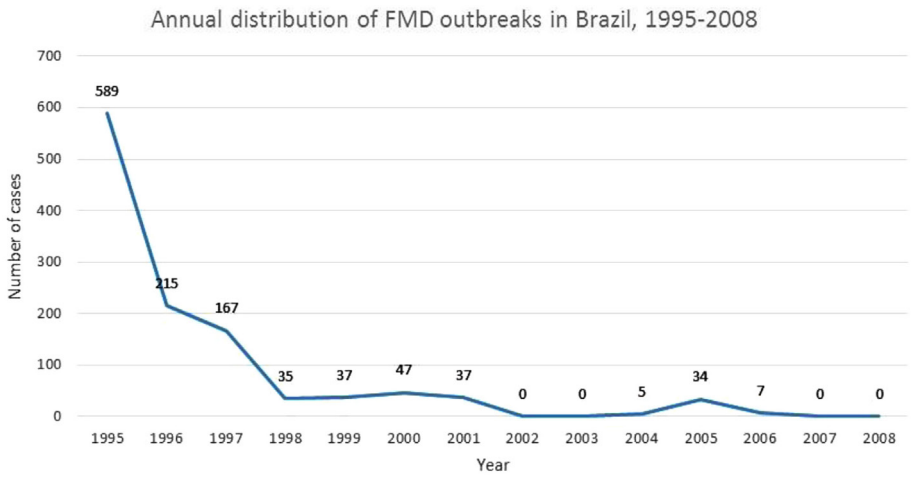

FIGURE 2 | Annual distribution of FMD outbreaks in Brazil, 1995–2008. Adapted from Departamento de Saude Animal (40).

in export, even the Amazonian states of Acre, Rondonia, Mato Grosso, and Tocantins. OIE-certified areas regressed in 2005, after FMD outbreaks in Mato Grosso do Sul, Parana, Mato Grosso, and Tocantins. In all likelihood, the problem began with initial infections in Mato Grosso do Sul, a lightly settled area near the Paraguayan border, where vaccinations were not always effectively administered. In any event, the outbreak areas were sanitized by 2008, and new lands, cleared for export, including a large part of the Amazonian State, Para (40).

FMD Policy Successes and Failures

Despite the dramatic drop in outbreaks depicted by **Figure 2**, Brazil's success in controlling FMD was slow in coming. There is little doubt that PNEFA played a decisive role in ultimately reducing FMD outbreaks, and features of the program (e.g., vaccination coverage and sanitary structures) have been shown to associate strongly with FMD control (37). That said, other factors outside the realm of policy intervention have played an important role in Brazil's experience with FMD, particularly in sustaining its long-run persistence. One of the early difficulties in sparking private sector commitment resided in a set of perverse incentives faced by large-scale ranchers. Given their early markets were primarily domestic, they saw little advantage in undertaking sanitary improvements vis-à-vis FMD, given costs associated with assembling large herds for regular vaccinations outweighed the potential losses from an FMD outbreak. A second reason that FMD control efforts remained ineffective through much of the twentieth century can be attributed to social and political turmoil, and associated economic difficulties such as inflation and poorly functioning credit markets. Only with currency reform under the *Plano Real* in the early 1990s did the macroeconomy stabilize sufficiently to engender a robust, agricultural expansion (39). Yet another reason that FMD proved resistant despite substantial initial efforts at control can be found in the wider experience of the South American continent, with its history of *caudilismo*, autocratic rulers, and corruption, all of which engendered a generalized distrust of government institutions. In the Brazilian case, this meant ranchers tended to avoid front-line organizations, such as the Veterinary Sanitary Service, a response that would stymie even the best-laid plans (34). The return to democracy in 1985 paved the way for new attitudes on part of the private sector and the population as a whole.

COMPUTATIONAL APPROACHES

The Brazilian case provides useful lessons for Kazakhstan, and Central Asia more generally, about how to combat FMD and Brucellosis in the interest of agricultural development. Thus, Kazakhstan is in the enviable position of being able to learn from someone else's successes, and failures. But that is not all, as Kazakhstani planners also have the advantage of being able to draw from a wide range of newly available computational approaches to epidemiology, with the potential to facilitate both the design and implementation of policy. Geospatial analysis, for example, has provided critical insight into the formation of animal disease clusters, and yielded descriptive and inferential assessments via spatial modeling (41). Clustering analyses comprise statistical approaches that test whether or not outbreak events are more spatially proximate than would be expected under randomized processes. Spatio-temporal analyses, such as accomplished by implementing a spatial scan statistic [see below; (42, 43)], evaluate if outbreaks (or other phenomena) cluster in both space and time, thereby shedding light on why clusters form, their size, and their duration. For the purposes of this article, we define spatial models as techniques that predict the spatial distribution of disease risk, or explain the spatio-temporal trends of outbreaks.

Clustering

AlKhamis et al. (44) applied a *direction test* within space-time clusters – defined using spatial scan statistics – to describe FMD transmission patterns across Israel in terms of size (square kilometer) and seasonality of outbreak clusters, and infection spread directions. Spatial scan statistics search for the most likely cluster of cases by placing varying sized circles over case locations and

comparing disease rates within and without the circles; circle sizes are varied up to the distance necessary for reaching a user defined maximum proportion of the population at risk. The temporal element is incorporated by a cylinder, with height measured in units of time (45), while directionality of cluster spread is established by a direction test, a two-dimensional mapping that calculates the average direction in which cases move, with significance computed by Monte Carlo simulation (46). Using an approach similar to AlKhamis et al. (44), Schlak (10) applied the direction test method to evaluate seasonal spread of FMD across the southernmost oblasts of Kazakhstan and showed significant linkage between outbreaks associated with seasonal peaks for the period 1955–1964. A study in Mongolia on Kazakhstan's eastern border employed the spatial scan statistic and the direction test to FMD data, and illustrated the direction in which outbreaks moved (47). Recently, Sytnik et al. (11) has applied kernel density estimation (KDE) to categorize risk nationally and has identified disease hotspots in southern Kazakhstan for the period 2011–2102. These recent hotspots tend to overlap with those identified by Schlak (10) for the period 1955–1964, over 60 years ago, suggesting outbreak areas of surprising persistence. Southern Kazakhstan borders Uzbekistan and Kyrgyzstan, each with a history of FMD; this highlights the trans-boundary nature of the problem for central Asia as a whole (48, 49).

Spatial Modeling

A growing number of studies apply spatial models to examine or predict the spread of animal diseases in a variety of geographic settings. For example, Lawson and Zhou (50) applied a Bayesian framework in evaluating biweekly count data at farm-scale to examine the 2001 UK FMD epidemic, a study that details the effects of disease control via vaccination and culling. Ward et al. (51, 52) employed geospatial simulation models to examine the role of wildlife in initiating livestock outbreaks in Texas. They showed that feral hogs (*Sus scrofa*) and white-tailed deer (*Odocoileus virginianus*) precipitated different cattle outbreak patterns, thereby providing insight how wildlife populations function as disease reservoirs, and how species spillovers can initiate livestock epidemics. The Texas study is particularly relevant to Kazakhstan, where *Saiga* antelope (*Saiga tatarica*) are highly sensitive to FMD and probably function as reservoirs for the disease. Using a spatio-temporal model, Morgan et al. (32) showed that virus spillover in either direction between antelope and livestock depends on *Saiga* migration timing and herd size. *Saiga* peak infection is greatest in spring and autumn, when calving and maternal immunity wear off, respectively. In light of the historical work by Schlak (10), FMD rates in cattle appear highest in southern Kazakhstan during the late summer and autumn months, a period that overlaps with *Saiga* spillover risk.

DISCUSSION

Computational modeling and analysis promise to help countries like Kazakhstan gain the epidemiological insight needed for controlling and eradicating biothreats, such as FMD and Brucellosis. As noted at the outset, Kazakhstan has taken a development path intent on using its pastoral resources to maximum extent by becoming a major beef exporter. This will require the shaping of relevant sanitary policy. The case study of Brazil indicates a broad set of factors that ultimately helped achieve control of a key biothreat, FMD, in pursuit of development objectives. Some of these reach far beyond responses based on the veterinary and epidemiological sciences, like the degree of societal trust in government institutions. For the Kazakhstani case, the transition to a market economy has been difficult overall, and many problems remain on the agricultural front, such as how to redistribute land and incentivize producers after ~60 years under a socialist government (20). While we appreciate the importance played by cultural and social context, we limit our remarks here to the technical side of the issue relating to veterinary policy vis-à-vis biothreats to livestock, and to the role of computational science in policy formulation. We do not consider important animal husbandry issues relating to herd structure (beef vs. dairy) and slaughter weight (22).

With a cattle herd of ~200,000,000 animals, distributed across ~2,700,000 rural properties, Brazil presents a challenging epidemiological case for reasons of sheer size. Adding to this are the disease vectors of the wild animal carriers found in its expansive, ecologically intact regions (31). Despite these daunting circumstances, the Brazilian government managed to control its FMD biothreat in only a few years, although success was long in the making. The PNEHA policy relied on a social compact involving state decentralization and public–private partnership. That said, the best administrative intentions make little headway without the commitment of financial resources. In the Brazilian case, these were substantial. PNEHA programmatic costs start out at about 100,000,000 $US in the early 1990s, and by 2008 they climb to nearly 450,000,000 $US, or about 2 $US per animal. These funds were spread across a large number of expenditure categories, including thousands of physical structures (e.g., surveillance posts along highways) and the creation of a dedicated labor force of ~2,500 veterinarians, together with support staff, both technical and administrative (40).

Political and social adjustments following the post-Soviet period have not been easy for Kazakhstan, and its economy continues to evolve with ongoing institutional reform. Nevertheless, with its herd of 6 million cattle, the biothreat to Kazakhstani livestock seems small in comparison to what Brazil faced only a few decades ago, at least from a numerical perspective. As already mentioned, Kazakhstan wishes to boost its current 1,000 metric tons of beef export to 180,000 by 2020 (2, 22). Given only 35% of existing pastures and hayfields are used, or about 630,000 km^2 out of 1,820,000 km^2, such an expansion would seem reasonable for a production system based on rangeland grazing (22). The export production target would generate nearly a billion dollars (720,000,000 $US) at current international beef prices (53). If we assume annual costs of FMD protection in Brazil at ~2 $US per animal, a herd on the order of 20,000,000 animals generates potential earnings far in excess of costs associated with OIE's SPS export requirements (54). Given Kazakhstan's willingness in advance to use earnings from its mineral and fuel exports to promote economic development in other sectors, the control of FMD as well as Brucellosis would appear to be within financial reach.

The preceding section suggests that geospatial technologies could be effectively implemented to help Kazakhstan best develop its pastoral resources. Such computational techniques were in their infancy when Brazil began concerted efforts to manage its FMD biothreat situation, so there is no prior experience to draw from in this regard. Nevertheless, from the applications to date, we argue that the right combination of spatio-temporal analyses – such as the direction test (44) and predictive modeling such as with "random forests" (55) – make possible the design of a spatially sensitized approach to the distribution of veterinary resources, one that could minimize costs and maximize revenues in terms of animal health and well-being. Although much of the computational research addresses FMD, geospatial program design would apply with equal force to other worrisome biothreats such as Brucellosis. Specific veterinary applications in the Kazakhstani case could:

1. Identify hotspots of disease clustering, in the interest of optimal spatial allocations of human resources for disease prevention and control. Similarly, identify "coldspots" to understand disease suppression mechanisms and environmental factors not conducive to FMD or Brucellosis infection. Use cluster sizes to define buffer zones within which to apply control measures during outbreak events (44).
2. Shape biodiversity configurations of the Kazakhstani landscape in order to ensure healthy domestic livestock and wildlife herds, given both populations may harbor diseases, with prevention less tenable in free ranging wildlife like the *Saiga* antelope. Identify natural buffers to disease transmission, and the optimal placement of fencing and other physical impediments to animal mobility.
3. Determine zones of disease likelihood in the interest of partitioning Kazakhstan into *circuits* with variable degrees of risk, as was done in Brazil, given OIE's acceptance of export from countries not entirely FMD-free. Expand from KDE to a probability-based prediction of disease risk (11).
4. Find conduits of disease transmission at region-scale and, together with geospatial information on transportation systems, isolate target points for the optimal placement of control structures. Use direction tests to assess needs for border control (44).
5. Analyze spatio-temporal outbreak patterns to develop a cost-minimal program of vaccination, including seasonal timing of vaccines (44).

We have focused our discussion on Kazakhstan as a site for the potential development of computational approaches to biothreat control. That said, it is important to note that the entire central Asian region would benefit both from an improved veterinary situation in Kazakhstan and from policy formulation that exploits the new powers of geospatial technology.

CONCLUSION

Biothreats continue to put livestock herds at risk worldwide, a circumstance with enormous implications for human welfare. Nowhere is this empirical observation more in evidence than for central Asia, where FMD and Brucellosis have long been endemic. For countries like Kazakhstan that once belonged to the FSU, institutional adjustments following its collapse have proved difficult, and presented challenges along a variety of fronts like that of building an economy that best serves its citizens. Kazakhstan, with an abundance of rangelands and deep cultural traditions of animal husbandry, seeks to secure its place in the global economy by improving opportunities for the export of animal products, notably beef. This reflects a perspicacious assessment of economic potential, but it will not be successful without a concerted effort to raise the health conditions of its livestock herds. The global economy provides opportunities for trade and foreign exchange earnings, but it is also a demanding task-master that places stringent demands on product safety relative to SPS standards.

The demands of the global economy can be met, however, as the case of Brazil testifies. Here, outbreaks of FMD fell precipitously in only a few years, once the Brazilian government pursued a public–private partnership that enabled the efficient decentralization of policy implementation. The loosening of OIE requirements – allowing for export from FMD-cleared zones within a country even if the biothreat persists elsewhere – dovetailed with Brazil's spatial approach to divide and conquer by partitioning its control efforts into *circuits*. Brazil's policy approach was not a silver bullet, given the importance of both institutional and economic change that ultimately provided the set of necessary background conditions enabling success. The parallels with Kazakhstan in this regard should provide grounds for encouragement, though. As Brazil moved away from a development model that inhibited market forces, and built new trust with its citizenry, its veterinary programs proved successful in a short period of time, enabling quick market consolidation and its number 2 rank as a global beef exporter. Kazakhstan presents a more difficult epidemiological case than Brazil given that policy to improve livestock health will have to fully integrate the wildlife factor, given conservationist concerns for the *Saiga* antelope, which may serve as livestock reinfection reservoirs. Luckily, the rapid evolution of geospatial technology provides a powerful new toolkit that can help governments like Kazakhstan bring development benefits to their peoples, in the face of analytical challenges to the design of policy.

REFERENCES

1. Maselli D, Inam-ur-Rahim. Setting the stage: key features of the present-day central Asian region: an introduction to the wider historical, social, political, economic, cultural, and ecologic contexts of the region in a nutshell. In: Squires V, editor. *Rangeland Stewardship, in Central Asia: Balancing Improved Livelihoods, Biodiversity Conservation and Land Protection*. Dordrecht: Springer (2012). p. 3–30.
2. Foster H. *KZN Tries Raising Beef to Move Economy*. USA Today (2014). Available from: http://www.usatoday.com/story/money/business/2014/06/05/kazakhstan-tries-raising-beef-to-moove-economy/10023747/ (Last accessed December 3, 2015)
3. Morgan N, Prakash A. International livestock markets and the impact of animal disease. *Rev Sci Tech* (2005) **25**:517–28.
4. Lundervold M, Milner-Gulland EJ, O'callaghan CJ, Hamblin C, Corteyn A, Macmillan AP. A serological survey of ruminant livestock in Kazakhstan

during post-Soviet transitions in farming and disease control. *Acta Vet Scand* (2004) **45**(3–4):211–24. doi:10.1186/1751-0147-45-211
5. Barnett PV, Carabin H. A review of emergency foot-and-mouth disease (FMD) vaccines. *Vaccine* (2002) **20**(11):1505–14. doi:10.1016/S0264-410X(01)00503-5
6. Reid SM, Ferris NP, Hutchings GH, Zhang Z, Belsham GJ, Alexandersen S. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J Virol Methods* (2002) **105**(1):67–80. doi:10.1016/S0166-0934(02)00081-2
7. Haydon DT, Kao RR, Kitching RP. The UK foot-and-mouth disease outbreak – the aftermath. *Nat Rev Microbiol* (2004) **2**(8):675–81. doi:10.1038/nrmicro960
8. Bessell PR, Shaw DJ, Savill NJ, Woolhouse ME. Statistical modeling of holding level susceptibility to infection during the 2001 foot and mouth disease epidemic in Great Britain. *Int J Infect Dis* (2010) **14**(3):e210–5. doi:10.1016/j.ijid.2009.05.003
9. Mamisashvili E, Kracalik IT, Onashvili T, Kerdzevadze L, Goginashvili K, Tigilauri T, et al. Seroprevalence of Brucellosis in livestock within three endemic regions of the country of Georgia. *Prev Vet Med* (2013) **110**(3):554–7. doi:10.1016/j.prevetmed.2012.12.005
10. Schlak AM. *Examining the Seasonality and Directionality of Foot-and-Mouth Disease Outbreaks in the Sheep Population in Southern Kazakhstan from 1955 to 1964*. Honors Thesis, University of Florida, Gainesville, FL (2013).
11. Sytnik I, Karibayev T, Tyulegenov S, Abenova A, Tashkenbayev A, Yerimbetov S. Surveillance of foot and mouth disease: a study of 2011-2012 outbreaks in Kazakhstan. *Ветеринарна медицина* (2013) **97**:51–4.
12. FAO. *Foot-and-Mouth Disease Situation Monthly Report June 2012* (2012). Available from: http://www.fao.org/fileadmin/user_upload/eufmd/docs/FMD_monthly_reports/June2012.pdf
13. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human Brucellosis. *Lancet Infect Dis* (2006) **6**(2):91–9. doi:10.1016/S1473-3099(06)70382-6
14. Lundervold M. *Infectious Diseases of Saiga Antelopes and Domestic Livestock in Kazakhstan*. Ph.D. thesis, University of Warwick, Coventry (2001).
15. Grushina T, Atshabar B, Syzdykov M, Daulbaeva S, Tserelson L, Kuznetsov A, et al. Universal indirect enzyme-linked immunosorbent assay for monitoring of human and animal Brucellosis in Kazakhstan. *Vaccine* (2010) **28**:F46–8. doi:10.1016/j.vaccine.2010.03.052
16. Jackson R, Ward D, Kennard R, Amirbekov M, Stack J, Amanfu W, et al. Papers & articles. *Vet Rec* (2007) **161**:476–82. doi:10.1136/vr.161.14.476
17. Porphyre T, Jackson R, Sauter-Louis C, Ward D, Baghyan G, Stepanyan E. Mapping Brucellosis risk in communities in the Republic of Armenia. *Geospat Health* (2010) **5**(1):103–18. doi:10.4081/gh.2010.191
18. Kerven C, Alimaev II, Behnke R, Davidson G, Malmakov N, Smailov A, et al. Fragmenting pastoral mobility: changing grazing patterns in post-Soviet Kazakhstan. Rangelands of Central Asia: Proceedings of the Conference on Transformations, Issues, and Future Challenges. Salt Lake City, UT: US Department of Agriculture, Forest Service, Rocky Mountain Research Station (2006). p. 99–110.
19. Pianciola N. The collectivization famine in Kazakhstan, 1931–1933. *Harv Ukr Stud* (2001) **25**(3–4):237–51.
20. Rahimon RM. Evolution of land use in pastoral culture in central Asia with special reference to Kyrgyzstan and Kazakhstan. In: Squires V, editor. *Rangeland Stewardship, in Central Asia: Balancing Improved Livelihoods, Biodiversity Conservation and Land Protection*. Dordrecht: Springer (2012). p. 51–66.
21. Robinson S, Milner-Gulland EJ. Political change and factors limiting numbers of wild and domestic ungulates in Kazakhstan. *Hum Ecol* (2003) **31**(1):87–110. doi:10.1023/A:1022834224257
22. Meldebekov S, Johnson SR. Can Kazakhstan be a successful player in the world beef market? *Life Sci J* (2014) **11**:289–93.
23. Bailey C, Convery I, Mort M, Baxter J. Different public health geographies of the 2001 foot and mouth disease epidemic: 'citizen' versus 'professional' epidemiology. *Health Place* (2006) **12**(2):157–66. doi:10.1016/j.healthplace.2004.11.004
24. Haydon DT, Chase-Topping M, Shaw DJ, Matthews L, Friar JK, Wilesmith J, et al. The construction and analysis of epidemic trees with reference to the 2001 UK foot-and-mouth outbreak. *Proc Biol Sci* (2003) **270**(1511):121–7. doi:10.1098/rspb.2002.2191
25. Woolhouse MEJ. Foot-and-mouth disease in the UK: what should we do next time? *J Appl Microbiol* (2003) **94**(s1):126–30. doi:10.1046/j.1365-2672.94.s1.15.x
26. Kitching RP, Hutber AM, Thrusfield MV. A review of foot-and-mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. *Vet J* (2005) **169**(2):197–209. doi:10.1016/j.tvjl.2004.06.001
27. Thompson D, Muriel P, Russell D, Osborne P, Bromley A, Rowland M, et al. Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. *Rev Sci Tech* (2002) **21**(3):675–85.
28. Petrick M, Oshakbaev D, Wandel J. *Kazakhstan's Wheat, Beef and Dairy Sectors: An Assessment of Their Development Constraints and Recent Policy Responses* (No. 145). Discussion Paper, Leibniz Institute of Agricultural Development in Central and Eastern Europe (2014). Halle.
29. Richardson NP. Export-oriented populism: commodities and coalitions in Argentina. *Stud Comp Int Dev* (2009) **44**(3):228–55. doi:10.1007/s12116-008-9037-5
30. McAlpine CA, Etter A, Fearnside PM, Seabrook L, Laurance WF. Increasing world consumption of beef as a driver of regional and global change: a call for policy action based on evidence from Queensland (Australia), Colombia and Brazil. *Glob Environ Change* (2009) **19**(1):21–33. doi:10.1016/j.gloenvcha.2008.10.008
31. Pinto AA. Foot-and-mouth disease in tropical wildlife. *Ann N Y Acad Sci* (2004) **1026**(1):65–72. doi:10.1196/annals.1307.008
32. Morgan ER, Lundervold M, Medley GF, Shaikenov BS, Torgerson PR, Milner-Gulland EJ. Assessing risks of disease transmission between wildlife and livestock: the *Saiga* antelope as a case study. *Biol Conserv* (2006) **131**(2):244–54. doi:10.1016/j.biocon.2006.04.012
33. Astudillo VM. A febre aftosa na América do Sul. *Hora Vet* (1992) **70**:16–21.
34. Mayen FL. Foot and mouth disease in Brazil and its control: an overview of its history, present situation and perspectives for eradication. *Vet Res Commun* (2003) **27**:137–48. doi:10.1023/A:1022863221356
35. Melo EC, López A. Control de la fiebre aftosa: la experiencia americana. *Rev Sci Tech Off Int Epiz* (2002) **21**(3):689–94.
36. Melo EC, Saraiva V, Astudillo V. Review of the status of foot and mouth disease in countries of South America and approaches to control and eradication. *Rev Sci Tech* (2002) **21**(3):429–33.
37. Müller CAS, de Mattos LB, de Lima JE. Determinantes da erradicação da febre aftosa no Brasil. *Org Rurais Agroindustriais* (2011) **9**(1):89–97.
38. Lyra TMP, Cortes JA. Controle de qualidade nos serviços públicos: a experiência dos programas de saúde animal. *Rev Educ Contin* (2002) **5**:320–31.
39. Walker R, Browder J, Arima E, Simmons C, Pereira R, Caldas M, et al. Ranching and the new global range: Amazônia in the 21st century. *Geoforum* (2009) **40**(5):732–45. doi:10.1016/j.geoforum.2008.10.009
40. Departamento de Saude Animal. *Programa Nacional de Erradicacao e Prevencao da Febre Aftosa: Relatorio Annual, 2008*. Brasilia: Ministerio da Agricultura, Pecuaria e Abastecimento (2009).
41. Kracalik IT, Blackburn JK, Lukhnova L, Pazilov Y, Hugh-Jones ME, Aikimbayev A. Analysing the spatial patterns of livestock anthrax in Kazakhstan in relation to environmental factors: a comparison of local (Gi*) and morphology cluster statistics. *Geospat Health* (2012) **7**(1):111–26. doi:10.4081/gh.2012.110
42. Kulldorff M. A spatial scan statistic. *Commun Stat Theory Methods* (1997) **26**(6):1481–96. doi:10.1080/03610929708831995
43. Barro AS, Kracalik IT, Malania L, Tsertsvadze N, Manvelyan J, Imnadze P, et al. Identifying hotspots of human anthrax transmission using three local clustering techniques. *Appl Geogr* (2015) **60**:29–36. doi:10.1016/j.apgeog.2015.02.014
44. AlKhamis MA, Perez AM, Yadin H, Knowles NJ. Temporospatial clustering of foot-and-mouth disease outbreaks in Israel and Palestine, 2006–2007. *Transbound Emerg Dis* (2009) **56**(3):99–107. doi:10.1111/j.1865-1682.2009.01066.x
45. Kracalik IT, Malania L, Tsertsvadze N, Manvelyan J, Bakanidze L, Imnadze P, et al. Evidence of local persistence of human anthrax in the country of Georgia associated with environmental and anthropogenic factors. *PLoS Negl Trop Dis* (2013) **7**(9):e2388. doi:10.1371/journal.pntd.0002388
46. Jacquez GM. *Stat!: Statistical Software for the Clustering of Health Events*. Ann Arbor, MI: Biomedware (1994).

47. Shiilegdamba E, Carpenter TE, Perez AM, Thurmond MC. Temporal-spatial epidemiology of foot-and-mouth disease outbreaks in Mongolia, 2000–2002. *Vet Res Commun* (2008) **32**(3):201–7. doi:10.1007/s11259-007-9018-6
48. Kesy A. Global situation of foot-and-mouth disease (FMD) – a short review. *Pol J Vet Sci* (2001) **5**(4):283–7.
49. FAO-EMPRES. *Foot-and-Mouth Disease: Situation Worldwide and Major Epidemiological Events in 2005-2006*. Uzbekistan (2007). Available from: http://www.fao.org/3/a-ai339e.pdf
50. Lawson AB, Zhou H. Spatial statistical modeling of disease outbreaks with particular reference to the UK foot and mouth disease (FMD) epidemic of 2001. *Prev Vet Med* (2005) **71**(3):141–56. doi:10.1016/j.prevetmed.2005.07.002
51. Ward MP, Laffan SW, Highfield LD. The potential role of wild and feral animals as reservoirs of foot-and-mouth disease. *Prev Vet Med* (2007) **80**(1):9–23. doi:10.1016/j.prevetmed.2007.01.009
52. Ward MP, Laffan SW, Highfield LD. Modelling spread of foot-and-mouth disease in wild white-tailed deer and feral pig populations using a geographic-automata model and animal distributions. *Prev Vet Med* (2009) **91**(1):55–63. doi:10.1016/j.prevetmed.2009.05.005
53. Morgan N, Tallard G. *Cattle and Beef International Commodity Profile*. Rome: Food and Agricultural Organization (2015).
54. Upton M. *Trade in Livestock and Livestock Products: International Regulation and Role for Economic Development*. Food and Agricultural Organization Livestock Information and Policy Branch. Livestock Policy Discussion Paper No. 6. Rome: Food and Agricultural Organization of the United Nations (2002).
55. Sehgal RNM, Buermann W, Harrigan RJ, Bonneaud C, Loiseau C, Chasar A, et al. Spatially explicit predictions of blood parasites in a widely distributed African rainforest bird. *Proc Biol Sci* (2011) **278**(1708):1025–33. doi:10.1098/rspb.2010.1720
56. Bekenov AB, Grachev IA, Milner-Gulland EJ. The ecology and management of the *Saiga* antelope in Kazakhstan. *Mamm Rev* (1998) **28**(1):1–52. doi:10.1046/j.1365-2907.1998.281024.x
57. Fry M. *The Status of the Saiga Antelope in the Ustiurt Region of Western Kazakhstan.* London: Doctoral dissertation, University of London (2004).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

PERSPECTIVE
published: 23 September 2015
doi: 10.3389/fpubh.2015.00215

Disruptive innovation can prevent the next pandemic

Affan T. Shaikh[1], Lisa Ferland[1], Robert Hood-Cree[2], Loren Shaffer[2] and Scott J. N. McNabb[1,3]*

[1] Public Health Practice, LLC, Atlanta, GA, USA, [2] Northrop Grumman Corporation, Falls Church, VA, USA, [3] Emory University, Atlanta, GA, USA

Public health surveillance (PHS) is at a tipping point, where the application of novel processes, technologies, and tools promise to vastly improve efficiency and effectiveness. Yet twentieth century, entrenched ideology and lack of training results in slow uptake and resistance to change. The term *disruptive innovation* – used to describe advances in technology and processes that change existing markets – is useful to describe the transformation of PHS. Past disruptive innovations used in PHS, such as distance learning, the smart phone, and field-based laboratory testing have outpaced older services, practices, and technologies used in the traditional classroom, governmental offices, and personal communication, respectively. Arguably, the greatest of these is the Internet – an infrastructural innovation that continues to enable exponential benefits in seemingly limitless ways. Considering the Global Health Security Agenda and facing emerging and reemerging infectious disease threats, evolving environmental and behavioral risks, and ever changing epidemiologic trends, PHS must transform. Embracing disruptive innovation in the structures and processes of PHS can be unpredictable. However, it is necessary to strengthen and unlock the potential to prevent, detect, and respond.

Keywords: disruptive innovation, public health surveillance, one health, public health surveillance strengthening, e-Surveillance, public health informatics

OPEN ACCESS

Edited by:
Nathan Wolfe, Metabiota, USA

Reviewed by:
Ronald Paul Rabinowitz, University of Maryland School of Medicine, USA
Nikkita Patel, University of Pennsylvania, USA

****Correspondence:***
Affan T. Shaikh, Public Health Practice, LLC, 2575 Peachtree Road NE, Atlanta, GA 30305, USA
ashaikh@publichealthpractice.com

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *20 June 2015*
Accepted: *31 August 2015*
Published: *23 September 2015*

Citation:
Shaikh AT, Ferland L, Hood-Cree R, Shaffer L and McNabb SJN (2015) Disruptive innovation can prevent the next pandemic. Front. Public Health 3:215. doi: 10.3389/fpubh.2015.00215

Introduction

Fifty-two years ago, Alexander Langmuir articulated our modern understanding of public health surveillance (PHS) – the systematic collection, consolidation and evaluation, and dissemination of data (1). In this workflow process, public health provides epidemiologic intelligence to assess and track conditions of public health importance, define public health priorities, evaluate programs, and conduct public health research (2). However, amid this rapidly changing world, PHS has remained sluggish and hindered by the impediments of siloed, vertical (outcome-specific) systems, inadequate training and technical expertise, different information and communication technology (ICT) standards, concerns over data sharing and confidentiality, poor interoperability, and inadequate analytical approaches and tools (3–7).

Gaps and impediments in PHS have become increasingly evident to the world in the wake of the largest Ebola epidemic ever – in which these challenges impacted our ability to prevent, detect, and respond. Under the looming threat of MERS-CoV, leishmaniasis, influenza, multidrug-resistant tuberculosis, and plague, the global public health community now realizes the urgent need to address shortcomings in PHS. Properly preparing for the next major outbreak hinges on our willingness to transform; the consequences of not doing so are dire.

Transforming PHS to meet the needs of the twenty-first century requires novel approaches. A helpful concept to understand and chart this future is *disruptive innovation* – a term first introduced by Clayton Christensen to describe innovations in technology and processes that disrupt existing markets (8). Disruptive innovations occur when advances in technologies or processes create markets in existing industries. This differs from sustaining innovations, where existing practices are incrementally improved to meet the demands of existing customers; in contrast, newly introduced innovations with disruptive potential (typically unrefined, simple, and affordable in character) target lower-end market needs or create entirely new market segments. As sustaining innovations improve disrupting technologies or processes, these new innovations will meet increasingly greater needs, capture greater market share, and eventually reshape the industry. Christensen uses the example of increasingly smaller disk sizes in the hard disk drive industry, the introduction of hydraulic technology in the mechanical excavator industry, and the rise of minimills in the steel industry to demonstrate the impact of disruptive innovations (8). Here, we describe the need for disruptive innovation in PHS and identify opportunities for disruption in PHS structures and processes.

Disruptive Innovation in Public Health Surveillance

To fulfill the Global Health Security Agenda and improve population health, PHS requires systematic improvements in planning and system design, data collection, data management, analysis, interpretation, dissemination, and program application. Numerous opportunities for disruption may affect any one of these activities. Taking stock of the challenges facing PHS in 2012, Thacker et al. described six concerns (9):

- complicated and heterogeneous lexicon
- expanding global surveillance networks to address evolving needs
- inadequate use of ICT tools
- lack of proper and comprehensive workforce development
- inconsistent data access and use
- poor data management, storage, and analysis practices

Disruptive technology can overcome these challenges. One example is the incorporation of digital tools to create electronic-based surveillance (e-Surveillance) – an ongoing disruption of paper-based methods. Adoption of the Internet enforced the need for standardization of vocabularies and opened doors for greater connectivity of local and global networks. Additionally, online training programs, e-Universities, and distance learning methods such as massive open online courses have opened unprecedented educational opportunities. Applications of ICT tools, such as Epi Info™ at the U.S. Centers for Disease Control and Prevention (CDC), have greatly improved data access, management, storage, and analysis practices. According to a 2012 Council of State and Territorial Epidemiologists assessment survey, health departments across the United States currently use a variety of notifiable diseases surveillance systems including custom-built systems, commercially available systems (e.g., Massachusetts Virtual Epidemiological Network, Scientific Technologies Corporation, Atlas, and Trisano), and the CDC National Electronic Telecommunications System for Surveillance (NETSS). As one of the first electronic systems to be developed in the early 1990s, NETSS uses a case-based structure (10, 11). However, as NETSS is restricted in functionality, a number of states continue to transition to the person- and standards-based National Diseases Surveillance System (NEDSS), a process that has been ongoing since 1998. NEDSS aims to integrate HIV/AIDS reporting systems, vaccination programs, and tuberculosis and other infectious disease tracking programs (10). Prior to NEDSS, these compartmentalized systems were isolated from one another due to differing data standards, legacy systems, and lack of tools for information exchange (10).

Disrupting Public Health Surveillance Structures

Demonstrated by the recent Ebola outbreak, the structure of PHS, including the various governance and collaborative frameworks guiding prevention, detection, and response, needs modernization. Foremost are the World Health Organization's (WHO) International Health Regulations (12), last revised in 2005. Meant to act as a safeguard against health threats by strengthening a country's capacity to detect, assess, respond, and report public health emergencies, its implementation where it is most needed falls short of achieving its purpose. One WHO report assessing implementation of the IHR (2005) in 2013 showed the African region to be well below global averages across all attributes measured, with no African state reporting full implementation (13, 14).

In the wake of the 2014 Ebola, successful compliance with the IHR (2005) requires much greater sustained investment in implementation (15). However, to meet evolving needs, this investment must be coupled with additional public health surveillance strengthening (PHSS). In early 2014, the United States of America in collaboration with 28 countries, the WHO, the Food and Agricultural Organization of the UN (FAO), and the World Organization for Animal Health (OIE) set forth to advance the IHR (2005) with the launch of the Global Health Security Agenda (16). The Agenda provides a renewed attempt to provide a framework and path with targets and milestones to accelerate progress in strengthening PHS.

New Frontiers

Public health surveillance strengthening requires disruption of governance and collaboration. While historically infectious disease centric, the scope of PHS has vastly expanded over recent years to include surveillance of chronic conditions and occupational hazards among many other public health issues. Furthermore, prevention, detection, and response are not restricted to national or regional health departments, as seen with the emergence of participatory PHS. Rather PHS is multisectoral, multilateral, and bidirectional. Recent years have given rise to new governmental, non-governmental, for-profit, and academic actors working at various levels (e.g., international, national, regional, and local) to fill gaps and meet needs while increasingly engaging the public.

With growing immediacy of the interaction between humans and animals, *One Health* has also emerged as a prerequisite for PHSS. With at least 60% of emerging and reemerging human infectious diseases being zoonotic, *One Health* unites human, veterinary, and environmental health disciplines for a more holistic approach to address the challenges we face (17). Leveraged for PHSS, *One Health* is a disruptive force in how we collaborate to prevent, detect, and respond to public health emergencies. A fully realized and integrated model of *One Health* PHS creates a proactive shift in prevention and response to the source – a disruption of existing, reactive PHS moving from outbreak to outbreak. However, achieving *One Health* PHS requires overcoming barriers. A review of *One Health* adoption by Uchtmann et al. highlighted underserved populations, professional barriers, incompatible vocabularies, sequestration of data, and territorial borders as impediments (18).

As *One Health* gains acceptance, the public health workforce will require interdisciplinary approaches to training. The CDC's Epidemic Intelligence Service (EIS) pioneered field epidemiology training. Accepting physicians, nurses, veterinarians, and persons with health science doctorates into the program indicates the growing acceptance of multidisciplinary PHS. Internationally, field epidemiology training programs (FETPs) offer robust solutions to the training needs of the public health workforce. The 55 accredited FETPs across the globe link to regional networks and the umbrella network known as the Training Programs in Epidemiology and Public Health Interventions Network (TEPHINET). They provide competency-based apprenticeships in applied epidemiology. FETP trainees have first-hand experiences responding to numerous cross-border and global public health investigations including disaster responses, noncommunicable diseases, and emerging or reemerging infectious disease threats. Together the EIS and FETP programs have trained over 6,980 public health professionals (19). This represents a critical resource for a poorly staffed workforce. With a trained workforce, innovative programs such as the U.S. Agency for International Development's Emerging Pandemic Threats program will have expertise to draw from across the animal and human health sectors to inform their PREDICT, PREVENT, IDENTIFY, and RESPOND projects and help build regional, national, and local *One Health* capacities for early disease detection, laboratory-based disease diagnosis, rapid response and containment, and risk reduction (20).

To accomplish robust IHR (2005) implementation and enhanced global health security, PHSS requires a well-trained public health workforce focused on *One Health* prevention in surveillance, epidemiology, laboratory, communications, and outbreak investigation. Developed countries must think globally and invest in developing countries' infrastructures and establish integrated PHS with proactive collaborative agreements to respond to public health emergencies. Enhanced governance frameworks, such as the Global Health Security Agenda, are critically important. Nigeria's prompt response and containment of the 2014 Ebola epidemic has been attributed to preexisting structures like a public health emergency operations center and available FETP trained epidemiologists, both targets of the Global Health Security Agenda (21).

Disrupting PHS Processes

Globalization has drastically changed our interactions with the biological world. A novel pathogenic infection discovered in one part of the globe can be easily carried thousands of miles away in a single day. Yet our implementation of the processes of PHS, including various advances in informatics and analytical tools, remains underutilized. As an example, the Integrated Disease Surveillance and Response (IDSR) regional framework, adopted in 1998 by the African regional office for the WHO, is a novel attempt at strengthening PHS capabilities at all levels in Africa (22). However, as recent studies reveal, this paper-based framework lacks timely reporting of PHS data and has been reported to be generally inefficient, error-prone, incomplete, and untimely (23). Disruptive innovation of the informatics and analytics used by PHS can address these gaps and impediments and improve IDSR. Developments in electronic health records, interoperability, information exchange, public information sharing, decision support, and cloud technologies are pushing ICT capabilities faster than PHS can evolve for the prevention, detection, and response. Once these advances are implemented, e-Surveillance can be fully realized and leveraged.

New Opportunities

Following sustained efforts toward PHSS, PHS can leverage novel, disruptive e-Surveillance approaches using informatics and analytics. Use of improved informatics techniques have been shown to improve completeness and timeliness of PHS data, but this depends critically on uniform standards of reporting, efficient workflow processes, and the willingness of practitioners to adopt disruptive technologies and processes (24, 25).

Disruptive innovations are not necessarily the most advanced technologies, but more often novel combinations of existing technologies or processes, offering simple and affordable alternatives. An emerging example of this sort of disruptive innovation in PHS is the emergence of participatory PHS with geographical information systems (GIS). Coupled with increasing availability and access to the Internet and mobile-based technologies, participatory PHS has also emerged in recent years as an innovative method of engaging the public and collecting regular, voluntary syndromic data (26). Examples of these PHS systems include ProMED-mail, Influenzanet, FluTracking, Reporta, Flu Near You, Dengue na Web, SaludBoricua, TuAnalyze, and Ushahidi. Common among these new PHS tools is an ability to aggregate, analyze, and visualize data in charts and maps in near real time while being freely accessible and easy to use.

Innovations in information aggregators for PHS leverage advances in the Internet and GIS. HealthMap, founded in 2006 by researchers at Boston Children's Hospital, combines various data from online news aggregators, eyewitness reports, expert-curated discussions, and validated official reports to map a unified and comprehensive view of the current global state of infectious diseases (26). While the free or low-cost nature is attractive and beneficial, the acceptability of these programs into mainstream systems depends greatly on their ability to enhance already existing data streams, something yet to be realized (27). As reported by Velasco et al., a number of vital issues must be addressed prior to

full integration including time-consuming and costly collaboration with statisticians, Internet and media experts, and computer scientists to work on components of data acquisition, data processing and filtering, personalization of results, and automation and verification of data (28). However, even with full integration, PHS must find a balance in supplementing these new technologies with existing PHS systems with official detection, verification, and validation responsibilities, using confidential sources. This underscores the need for PHSS before implementation of e-Surveillance.

The increasing prevalence of mobile and wireless technologies, recognized for their potential impact on health by the WHO in 2011, also offers a unique opportunity for disruption of PHS processes (29). With rapid technological development, falling market prices, increasing network coverage, and explosive user growth, the developing world has the greatest to gain from the implementation of mobile and wireless health technologies (30). Mobile networks are particularly valuable when, considering in some parts of the world, mobile penetration has outpaced other advanced communication technologies, extending far beyond the electrical grid and health infrastructure in some instances (31).

However, despite pervasive attributes, technological inequalities remain an important consideration. Among cell phone users today in the United States, African Americans and Hispanics are more likely to look up health information using a mobile device than are White non-Hispanics (32). In Brinkel et al.'s review of mobile health practices for PHS in Sub-Saharan Africa, PHS with real-time and validated data was strongly needed to strengthen disease monitoring capacity. However, mobile phone-based projects in PHS continue to be small-scale and fragmented (31). The success of mobile health projects generally correlates with their accessibility, acceptance, adaptation to local contexts, cost of the technology, stakeholder collaboration, and government involvement (33).

Discussion

The desire for comprehensive PHS with interoperable electronic systems and data captured from many sources and across many diseases is not new but is still far from being realized (34). Disrupting the structures and processes of PHS with novel technologies and processes will help achieve this vision. Strong and sustained investments in PHSS leveraging opportunities in One Health and applied epidemiology training programs will bolster the structures for prevention and response to public health emergencies. As PHSS takes hold, innovative approaches toward e-Surveillance, like participatory systems and mobile health, can be leveraged to drastically improve detection of public health emergencies. The developing world, with limited resources and infrastructure capabilities, and less access to higher market end traditional PHS systems, stand to benefit the greatest from these disruptions. Together, disruptions in structures and processes leading to PHSS and e-Surveillance promise to transform current practice; a new vision emerges where the PHS workforce implements the latest technologies and processes, and the information required to make informed decisions is available when it is needed, where it is needed.

References

1. Langmuir AD. The surveillance of communicable diseases of national importance. *N Engl J Med* (1963) **268**:182–92. doi:10.1056/NEJM196301242680405
2. Thacker SB. Historical development. In: Lee L, Teutsch S, Thacker S, St Louis M, editors. *Principles and Practice of Public Health Surveillance*. New York, NY: Oxford University Press (2000). p. 1–15.
3. Carroll LN, Au AP, Detwiler LT, Fu TC, Painter IS, Abernethy NF. Visualization and analytics tools for infectious disease epidemiology: a systematic review. *J Biomed Inform* (2014) **51**:287–98. doi:10.1016/j.jbi.2014.04.006
4. Frenk J, Chen L, Bhutta ZA, Cohen J, Crisp N, Evans T, et al. Health professionals for a new century: transforming education to strengthen health systems in an interdependent world. *Lancet* (2010) **376**:1923–58. doi:10.1016/S0140-6736(10)61854-5
5. Guilbert JJ. The World Health Report 2006: working together for health. *Educ Health (Abingdon)* (2006) **19**:385–7. doi:10.1080/13576280600937911
6. Jerolmack C. Who's worried about turkeys? How 'organisational silos' impede zoonotic disease surveillance. *Sociol Health Illn* (2013) **35**:200–12. doi:10.1111/j.1467-9566.2012.01501.x
7. Savel TG, Foldy S. The role of public health informatics in enhancing public health surveillance. *MMWR Morb Mortal Wkly Rep* (2012) **61**:20–4.
8. Christensen C. *The Innovator's Dilemma: When New Technologies Cause Great Firms to Fail*. New York, NY: Harper Business (2013). 296 p.
9. Thacker SB, Qualters JR, Lee LM. Public health surveillance in the United States: evolution and challenges. *MMWR Morb Mortal Wkly Rep* (2012) **61**:3–9.
10. CDC. State electronic disease surveillance systems – United States, 2007 and 2010. *MMWR Morb Mortal Wkly Rep* (2011) **60**:1421–3.
11. CDC. National electronic telecommunications system for surveillance – United States, 1990-1991. *MMWR Morb Mortal Wkly Rep* (1991) **40**:502–3.
12. WHO. International Health Regulations (2005).
13. Kasolo F, Tshioko FK, Impouma B, Yahaya AA, Bakyaita N, Gaturuku P, et al. Implementation of the International Health Regulations (2005) in the African region. *Afr Health Obs* (2013) **18**:11–3.
14. WHO. *Summary of States Parties 2013 Report on IHR Core Capacity Implementation: Regional Profiles*. Geneva: WHO (2014).
15. Ebola: what lessons for the International Health Regulations? *Lancet* (2014) **384**:1321. doi:10.1016/S0140-6736(14)61697-4
16. Frieden TR, Tappero JW, Dowell SF, Hien NT, Guillaume FD, Aceng JR. Safer countries through global health security. *Lancet* (2014) **383**:764–6. doi:10.1016/S0140-6736(14)60189-6
17. Taylor LH, Latham SM, Woolhouse ME. Risk factors for human disease emergence. *Philos Trans R Soc Lond B Biol Sci* (2001) **356**:983–9. doi:10.1098/rstb.2001.0888
18. Uchtmann N, Herrmann JA, Hahn EC III, Beasley VR. Barriers to, efforts in, and optimization of integrated one health surveillance: a review and synthesis. *Ecohealth* (2015) **1**:17. doi:10.1007/s10393-015-1022-7
19. Subramanian RE, Herrera DG, Kelly PM. An evaluation of the global network of field epidemiology and laboratory training programmes: a resource for improving public health capacity and increasing the number of public health professionals worldwide. *Hum Resour Health* (2013) **11**:45. doi:10.1186/1478-4491-11-45
20. Morse SS. Public health surveillance and infectious disease detection. *Biosecur Bioterror* (2012) **10**:6–16. doi:10.1089/bsp.2011.0088
21. Heymann DL, Chen L, Takemi K, Fidler DP, Tappero JW, Thomas MJ, et al. Global health security: the wider lessons from the west African Ebola virus disease epidemic. *Lancet* (2015) **385**:1884–901. doi:10.1016/S0140-6736(15)60858-3
22. McNabb SJ, Chungong S, Ryan M, Wuhib T, Nsubuga P, Alemu W, et al. Conceptual framework of public health surveillance and action and its application in health sector reform. *BMC Public Health* (2002) **2**:2. doi:10.1186/1471-2458-2-2

23. Pascoe L, Lungo J, Kaasbøll J, Koleleni I. Collecting integrated disease surveillance and response data through mobile phones. In: Cunningham P, Cunningham M, editors. *IST-Africa 2012: Conference Proceedings*. Dar es Salaam: A Union (2012). p. 6.
24. Iqbal K, Klevens RM, Jiles R. Comparison of acute viral hepatitis data quality using two methodologies, 2005-2007. *Public Health Rep* (2012) **127**:591–7.
25. CDC. Progress in improving state and local disease surveillance – United States, 2000-2005. *MMWR Morb Mortal Wkly Rep* (2005) **54**:822–5.
26. Wojcik OP, Brownstein JS, Chunara R, Johansson MA. Public health for the people: participatory infectious disease surveillance in the digital age. *Emerg Themes Epidemiol* (2014) **11**:7. doi:10.1186/1742-7622-11-7
27. Keller M, Blench M, Tolentino H, Freifeld CC, Mandl KD, Mawudeku A, et al. Use of unstructured event-based reports for global infectious disease surveillance. *Emerg Infect Dis* (2009) **15**:689–95. doi:10.3201/eid1505.081114
28. Velasco E, Agheneza T, Denecke K, Kirchner G, Eckmanns T. Social media and internet-based data in global systems for public health surveillance: a systematic review. *Milbank Q* (2014) **92**:7–33. doi:10.1111/1468-0009.12038
29. WHO. *Atlas eHealth Country Profiles: Based on the Findings of the Second Global Survey on e-Health*. Geneva: WHO (2011).
30. Piette JD, Lun KC, Moura LA Jr, Fraser HS, Mechael PN, Powell J, et al. Impacts of e-health on the outcomes of care in low- and middle-income countries: where do we go from here? *Bull World Health Organ* (2012) **90**:365–72. doi:10.2471/BLT.11.099069
31. Brinkel J, Kramer A, Krumkamp R, May J, Fobil J. Mobile phone-based mHealth approaches for public health surveillance in sub-Saharan Africa: a systematic review. *Int J Environ Res Public Health* (2014) **11**:11559–82. doi:10.3390/ijerph111111559
32. Fox S, Duggan M. *Mobile Health 2012*. Washington, DC: Pew Internet & American Life Project (2012).
33. Aranda-Jan CB, Mohutsiwa-Dibe N, Loukanova S. Systematic review on what works, what does not work and why of implementation of mobile health (mHealth) projects in Africa. *BMC Public Health* (2014) **14**:188. doi:10.1186/1471-2458-14-188
34. Thacker SB, Stroup DF. The future of national public health surveillance in the United States. *J Public Health Manag Pract* (1996) **2**:1–3. doi:10.1097/00124784-199623000-00003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

MINI REVIEW
published: 30 November 2015
doi: 10.3389/fpubh.2015.00251

Reassessing Biological Threats: Implications for Cooperative Mitigation Strategies

Summer Elise Galloway[†], *Stephanie Rachel Petzing*[†] *and Catharine Grace Young*[*†]

Department of Defense, American Association for the Advancement of Science and Technology, Washington, DC, USA

Multiple factors ranging from globalization to ecosystem disruption are presenting the global community with evolving biological threats to local, national, and global security that reach beyond the realm of traditional bioweapon threats. As a result, mitigation strategies have adapted necessarily to the increased diversity of biological threats. In general, response and preparedness strategies have largely shifted from being primarily reactive to traditional biological weapons to more proactive in nature. In this review, we briefly explore biological threats through a wider aperture, to embrace a greater appreciation of viral pathogens, antimicrobial resistance, and agricultural pathogens, and their potential to cause civil, economic, and political devastation. In addition, we discuss current mitigation strategies codified by the Global Health Security Agenda and the One Health paradigm as well as some of the available tools to assist with their sustainable implementation.

Keywords: biological threats, mitigation strategies, Global Health Security Agenda, One Health, Cooperative Threat Reduction Program, Department of Defense, antimicrobial resistance, biosurveillance

OPEN ACCESS

Edited by:
Hillary H. Carter, U.S. Department of State, USA

Reviewed by:
Gregory Koblentz, George Mason University, USA
Rita Sue Guenther, The National Academy of Sciences, USA

****Correspondence:***
Catharine Grace Young
catharine.g.young.ctr@mail.mil
[†]Summer Elise Galloway, Stephanie Rachel Petzing and Catharine Grace Young have contributed equally to this work.

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *16 June 2015*
Accepted: *26 October 2015*
Published: *30 November 2015*

Citation:
Galloway SE, Petzing SR and Young CG (2015) Reassessing Biological Threats: Implications for Cooperative Mitigation Strategies. Front. Public Health 3:251. doi: 10.3389/fpubh.2015.00251

INTRODUCTION

The world has entered a new era of biological threats due to unprecedented changes brought by globalization, growing agricultural demands, the diffusion of advanced biotechnologies, and insufficient reporting of outbreaks. Outbreaks of zoonotic diseases for which adequate treatments or vaccinations are unavailable or of diseases that could cripple the agriculture sector are examples of "non-traditional" biological threats with the potential to cause public health and economic devastation. Although these threats fall outside the traditional boundaries of bioterrorism, they have become a major target of the biodefense community in order to protect U.S. Armed Forces and citizens at home and abroad as well as our allies. Mitigating these threats requires the close cooperation of the national security, public health, and agriculture communities within the United States, partner nations, international organizations, and non-government organizations.

This article provides an overview of the non-traditional biological threats posed by emerging viruses, antibiotic-resistant bacteria, and agricultural pathogens. The article then describes efforts by the government sector [e.g., the Global Health Security Agenda (GHSA)] and the non-government sector (e.g., the One Health paradigm) to develop frameworks to address these threats. Finally, the article offers an assessment of the contribution that the Cooperative Biological Engagement Program (CBEP) within the Department of Defense's Cooperative Threat Reduction (CTR) program has made to global health security through its programs to strengthen biosurveillance (BSV) and building partner capacity through collaborative research.

VIRAL THREATS

While viruses are considered traditional biological threats by the defense and security sectors, they are typically discussed in terms of bioengineering to increase pathogenicity or "weaponize" rather than appreciating the threat they pose naturally. In this regard, an understanding of the health care system, cultural practices of the affected population, and political and economic climate of the affected region are critical and often underestimated aspects of dealing with viral outbreaks. Even when these factors are understood, the capabilities of governments and non-government organizations to respond effectively are complex and legitimate.

There are a number of viral threats to public health as well as regional, national, and global health security. However, the overwhelming majority of viruses on the Centers for Disease Control and Prevention and the United States Department of Agriculture "Select Agents" lists are RNA viruses, which are among the most serious uncontrolled causes of extant and emerging infectious diseases (1, 2). Owing to the nature of their replication machinery, which lacks proofreading activity and therefore has high error rates, RNA viruses exist as quasi-species or a "molecular swarm" of viral genomes. This leads to each having different levels of fitness within the population and positioned to respond readily to selective pressure (3–5). This feature affords RNA virus populations the capability to evolve at rates of up to one million times faster than their DNA counterparts, presenting a daunting public health challenge when designing therapeutics and vaccines. This is exemplified by the antiviral resistance in HIV and influenza virus populations, as well as the evolution of influenza A virus, which necessitates the annual reformulation of the vaccine. Other more genetically stable viruses, such as the DNA virus causing smallpox, have also been considered traditional biological threats. However, due to the availability of an effective vaccine, the success of the eradication campaigns over the last 45 years, and the consolidation of virus stocks, smallpox is generally disregarded as a public health threat. It has been speculated that due to the cessation of mass vaccination in 1980 and waning immunity, less than 20% of the population is sufficiently protected from infection (6). As such, the result of an outbreak of smallpox, either accidental or intentional, could be devastating to a regional, national, or global population.

The recent Ebola virus (an RNA virus that causes hemorrhagic fever) epidemic in West Africa highlights the civil, economic, and public health system destabilization that can occur as the result of a naturally occurring epidemic, let alone an intentional outbreak. For example, the epidemic has resulted in over 27,000 reported cases and more than 11,000 deaths, with an economic impact on Guinea, Sierra Leone, and Liberia rising to approximately 5% of their combined GDP in 2014 and 12% in 2015 (7). This outbreak, along with the potential for other similar catastrophic events, illustrates that having effective global BSV capabilities and response plans in place are crucial in mitigating viral pathogen threats.

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance is appreciated in the public health arena as an emerging threat, but is only beginning to be explored in the biological defense sector. The threat of especially dangerous pathogens, which have either naturally or through genetic engineering acquired resistance to vaccines or antimicrobials, is severe (8). Genes conferring resistance can be carried on mobile genetic elements (e.g., plasmids), which can be inconspicuously maintained in less concerning bacterial species and transferred through natural mechanisms to pathogenic agents (9). This is illustrated by a natural plague outbreak in Madagascar (1995), where a patient was infected with a plague isolate that was resistant to streptomycin, chloramphenicol, tetracycline, sulfonamides, ampicillin, kanamycin, and spectinomycin, all of which are first- and second-line drugs used to treat and prevent plague (10). Further investigation revealed that the genes for this alarming pattern of resistance were carried on a plasmid originating in the Enterobacteriaceae family, which was able to be transferred from *Escherichia coli* to the plague bacterium. This demonstrates the real possibility of dangerous pathogens obtaining antimicrobial resistance genes from innocuous bacteria in the environment. This evolving threat has received highest attention with the issuance of Executive Order 13676: "Combating Antibiotic-Resistant Bacteria" by President Barack Obama in September 2014 (11) and a corresponding "National Action Plan for Combating Antibiotic-resistant Bacteria" released in March 2015 (12).

AGRICULTURAL PATHOGENS

Despite the agriculture sector playing a formidable role in the economic, social, and political stability of the U.S., it has yet to receive the needed attention with respect to protection against major biological threats. Unlike the developing world, if a devastating outbreak of either animal or plant disease occurred in the U.S. (e.g., African swine fever, soybean rust, and potato wart), it would be the severe economic consequences that would pose as the greatest threat, not famine (13). A powerful example is the case of foot-and-mouth disease (FMD) outbreak in the United Kingdom (2001), a country previously free of the disease. FMD is a viral disease that affects cloven-hoofed animals, and while it is a disease of low mortality, it is highly infectious and spreads via aerosols or direct contact with contaminated equipment or feed. Following the first outbreak, the European Union blocked imports of British beef, sheep, and swine, leading to an unprecedented loss of revenue of approximately $13 billion and the culling of approximately 10 million cattle and sheep. For the U.S., annual sales of beef are in the realm of $40 billion, illustrating that the trade consequences of an FMD outbreak could be just as devastating. Surprisingly, the impact in countries deemed FMD-free has received more attention than the impact of outbreaks in FMD-endemic countries, despite the annual impact of FMD-related losses, estimated to be up to $21 billion. As with public health threats, mitigation of agriculture threats face many of the same obstacles and requires a well-coordinated, timely, and robust animal disease-reporting system. In addition, worldwide control of agricultural pathogens requires coordination within and between countries requiring both national and international public investment.

It is clear from the global spread and economic destruction of infectious diseases such as Ebola and FMD and the emergence of antimicrobial resistance pathogens that a new approach to combat

global biological threats is required. Increased globalization of trade and travel has resulted in an international community that is not secure from biological threats. Importantly, more than 80% of countries did not meet the World Health Organization deadline for being prepared for infectious disease threats. Severe Acute Respiratory Syndrome (SARS) in 2003 is a primary example of this. In under a year, SARS swept 29 countries, was diagnosed in over 8000 patients, and led to 774 deaths (14). The rapid spread of SARS was made possible by the unprecedented volume and speed of international travel and the inability of countries to handle the detection, diagnosis, and reporting of the disease.

GLOBAL HEALTH SECURITY AGENDA

To tackle such issues, the GHSA, a new multisectoral and interagency government approach to combat global threats, was launched by the U.S. and international partners in 2014. The major objectives of the GHSA are to prevent avoidable epidemics, detect threats early, and respond rapidly and effectively on a global scale. Incidents such as the SARS outbreak highlight that one nation cannot achieve global health security on its own. As such, the U.S. has committed to working with 30 countries over the next 5 years to implement the GHSA. This strategic approach is aimed to specifically strengthen disease response capabilities and rapidly detect and improve transparency in outbreak reporting by supporting existing agreements under the World Health Organization International Health Regulations 2005 (15), the World Organization for Animal Health Codes (16), and the Codex Alimentarius International Food Standards (17). In short, the GHSA is a collaborative effort to secure the world from global health threats posed by infectious diseases, whether naturally occurring, or the result of an accidental or intentional release of pathogens.

ONE HEALTH

Estimates indicate that approximately 75% of emerging or reemerging infections are vector-borne or zoonotic. Within the last 20 years, there have been several major instances of cross-species transmission that have caused severe public health, economic, and political consequences, not to mention effects on public confidence in the ability of governments to respond to emerging biological threats. Examples include the transmission of H5N1 to humans that was first reported in 1997 (18, 19); the West Nile virus outbreak in 1999 that possibly originated from illicit animal importation into New York (20); the SARS coronavirus (CoV) epidemic in 2003 originating from bats and/or civets (21, 22); the H1N1 influenza A virus pandemic of 2009 originating from swine (23); the Middle East Respiratory Syndrome CoV epidemic in 2012 that likely originated from bats and/or camels (24, 25); the transmission of H7N9 IAV to humans in 2013 from poultry (26); and the most recent Ebola virus epidemic in 2014 that was likely transmitted to humans by bats. These examples underscore how devastating zoonotic diseases can be, even if rapidly, detected and geographically contained.

The concept of "One Health" is not new, as practitioners have long recognized the connection between animal and human disease, but it has achieved greater traction among the human and animal public health sectors in recent years. The American Veterinary Medical Association defines One Health, in broad terms, as "the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and the environment."(27). Achieving this goal is articulated by the One Health Initiative that includes several joint efforts in: (1) the education of stakeholders; (2) communication in journals, conferences, and health networks; (3) clinical care through assessment, treatment, and prevention of cross-species transmission; (4) implementing disease surveillance systems; (5) achieving a better understanding of factors contributing to zoonotic transmission; (6) the development and evaluation of new diagnostics, therapeutics, and vaccines; and (7) the education of leaders and the public sector through responsible journalism (28).

There are undeniable benefits to operating under the One Health umbrella, but consideration of unintentional consequences is worth noting. For example, how should the economic implications of reporting outbreaks in animal populations balance against public health? Are there mechanisms in place to reduce the severity of disruptions to a fledgling economy to bolster reporting levels? Regardless of the barriers, what is clear is that achieving the vision of the One Health Initiative will require substantial and sustained international commitment in terms of funding, research, public and animal health capacity building, and infrastructure development.

THE COOPERATIVE THREAT REDUCTION PROGRAM

The CTR Program has evolved from the original Nunn-Lugar CTR program, which focused on materials and expertise left behind by the Soviet regime, into a comprehensive program to reduce worldwide threats from nuclear, radiological, chemical, and biological weapons. A central tenet of the CTR Program is sustainability, as the ultimate vision is that partner countries have the appropriate facilities that incorporate safety and security standards, necessary training, and technical expertise to effectively mitigate nuclear, chemical, and biological threats within their borders.

Within CTR, CBEP engages with partner country governments, institutions, and scientists to reduce the threat from traditional biowarfare agents, as well as agents that do not have a history of attempted weaponization, but are of security concern due to their potential to cause mortality, economic, or civil disruption. As the nature of biological threats evolves, CBEP has demonstrated the flexibility and agility to address the challenge, by encouraging the safe and secure development of biological research and scientific workforce capacity. Below, we focus on two major mechanisms for accomplishing the CTR mission: biosurveillance, which inherently incorporates biosafety and biosecurity elements, and the Cooperative Biological Research (CBR) program within CBEP.

BIOSURVEILLANCE

Biosurveillance refers to the continual process of gathering, analyzing, and interpreting data in order to achieve early detection, warning, and awareness of biological threats to human or animal health as well as to national, regional, and global security. BSV capability requires trained epidemiologists and clinicians,

laboratory capability to diagnose disease, and information systems to manage and relay disease information to decision makers responsible for managing outbreak responses. The foundation of a functioning BSV system is a capable network of local laboratories that serve as spokes to a central reference laboratory for diagnostic and reporting purposes. Together with host governments, the U.S. interagency, and non-government organizations, CBEP improves capabilities that support sustainable and integrated laboratory networks in partner countries. Notable benefits of engaging partner countries on BSV-related efforts are the overall safety and security culture that may be imparted on professionals in the BSV network and by investing in coordinated surveillance of human, animal, and plant health, not only does overall health improve, but practitioners have an increased ability to detect outbreaks of pathogens of security concern, which may lead to more timely implementation of mitigation strategies.

Cooperative Biological Engagement Program employs several core strategies in working with a partner country to enhance BSV capacity and capability, including building secure laboratory infrastructure and identifying and training laboratory personnel and clinicians to conduct safe and secure diagnostics, especially when working with dangerous and highly infectious pathogens. CBEP also supports the implementation of BSV-based research to monitor endemic diseases in order to differentiate between natural, accidental, and deliberate outbreaks, as well as information system networks to manage health-related data, critical to BSV, for the purpose of analysis and reporting to the authorities responsible for implementing specific biological mitigation strategies.

The implementation of a BSV system can be complicated; there is no universal solution, and multiple factors should be considered in order to strike the appropriate balance between specificity and sensitivity. These include the cost of implementation, most effective type of BSV that should be implemented for a given country or disease (active vs. passive and syndromic vs. laboratory surveillance), most effective information and analysis system to use, laboratory capabilities of the country, and extent of known biological markers of disease. The assessment of these factors requires a collaborative and interdisciplinary approach ranging from engagement with public and animal health practitioners to coordination with government officials and law enforcement. Considering the gamut of collaboration, CBEP is uniquely positioned to affect a multitude of capabilities that ultimately lead to improved local, national, and global health security.

COOPERATIVE BIOLOGICAL RESEARCH

As illustrated by the Ebola outbreak in West Africa, a naturally occurring outbreak can quickly become a transcontinental threat, the mitigation of which requires a modern approach. This includes building networks of professionals trained in the safe and secure conduct of biological research and surveillance. CBEP's CBR funds collaborative projects with researchers from partner countries. The projects include elements of biosafety and biosecurity training, and the research topics are strategic, furthering the interests of the partner countries as well as the objectives of CBEP. These efforts contribute to global BSV of human and animal pathogens endemic in partner countries, providing a baseline prevalence of biological disease threats within a geographic area. Understanding the incidence and natural variations of pathogens in an ecosystem is crucial to the investigation of an unusual outbreak and to the public, animal, and/or plant health response. These data help to determine the source of an outbreak as well as make a rapid determination of whether an increase in infections or antimicrobial resistance is natural or due to an accidental or intentional release. The cooperative nature of the CBR program provides a platform for the international collaborative work necessary to address transboundary threats such as zoonotic diseases carried across borders by infected humans, importation of animals, or natural movement of mobile vectors such as bats. These diseases require a comprehensive One Health approach involving both human and animal health sectors, which CBR facilitates.

Moreover, the scientific collaboration encouraged by the CBR program is an example of the diplomacy and engagement that fosters continued communication networks among the international community that are crucial during outbreaks of pathogens. The language of science is universal, and the value of international collaboration is clear to those engaged in scientific research. Scientists welcome shared information regarding data, protocols, equipment, and training, and interactions of this nature are excellent starting points for U.S. engagement, as well as collaboration among partner countries, facilitating peaceful collaboration and free exchange of information.

CONCLUSION

The world is constantly changing: humans across the globe are more interconnected and, due to habitat encroachment and ecological shifts, are in closer and more frequent contact with animal reservoirs of disease than ever before. This combined with free information flow, rapid scientific development, and the ever-present potential of misuse of biological agents presents a new and challenging environment for biological threat reduction requiring an adapted strategic approach. Open, effective, and consistent international collaboration for surveillance of pathogens is a backbone of this new approach. It must be comprehensive and include emerging threat agents, which are not necessarily limited to the traditional bioweapon agents of the Cold War era. Safe, secure, and sustainable capacity development in partner countries to increase the network of trained biological science professionals, as well as interconnected and effective infrastructure, is necessary. CBEP combines international agility and experiences with cutting-edge technical expertise to not only effectively reduce the threat from "traditional" bio-warfare agents but also emerging biological threats.

ACKNOWLEDGMENTS

The authors thank Mike Mahar for his helpful discussion and critical comments. The authors would like to acknowledge support for this work from Oak Ridge Institute for Science and Education (ORISE), American Association for the Advancement of Science (AAAS) and the Threat Reduction and Arms Control (TRAC) Office of the Department of Defense (DoD).

REFERENCES

1. Federal Select Agent Program. *Select Agents and Toxins List* (2015). Available from: http://www.selectagents.gov/SelectAgentsandToxinsList.html
2. Domingo E. Mechanisms of viral emergence. *Vet Res* (2010) **41**:38. doi:10.1051/vetres/2010010
3. Steinhauer DA, dela Torre JC, Holland JJ. High nucleotide substitution error frequencies in clonal pools of vesicular stomatitis virus. *J Virol* (1989) **63**:2063–71.
4. Arias A, Arnold JJ, Sierra M, Smidansky ED, Domingo E, Cameron CE. Determinants of RNA-dependent RNA polymerase (in)fidelity revealed by kinetic analysis of the polymerase encoded by a foot-and-mouth disease virus mutant with reduced sensitivity to ribavirin. *J Virol* (2008) **82**:12346–55. doi:10.1128/JVI.01297-08
5. Domingo E, Sheldon J, Perales C. Viral quasispecies evolution. *Microbiol Mol Biol Rev* (2012) **75**:159–216. doi:10.1128/MMBR.05023-11
6. Henderson DA. The looming threat of bioterrorism. *Science* (1999) **283**:1279–82. doi:10.1126/science.283.5406.1279
7. World Bank Group. *The Economic Impact on Sub-Saharan Africa: Updated Estimates for 2015* (2015). Available from: https://openknowledge.worldbank.org/bitstream/handle/10986/21303/937210REVISED000Jan02002015000FINAL.pdf?sequence=1
8. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* (2015) **13**:42–51. doi:10.1038/nrmicro3380
9. Yamashita A, Sekizuka T, Kuroda M. Characterization of antimicrobial resistance dissemination across plasmid communities classified by network analysis. *Pathogens* (2014) **15**:356–76. doi:10.3390/pathogens3020356
10. Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, et al. Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. *N Engl J Med* (1997) **337**:677–81. doi:10.1056/NEJM199709043371004
11. *Executive Order 13676: "Combating Antibiotic-Resistant Bacteria"* (2014). Available from: https://www.whitehouse.gov/the-press-office/2014/09/18/executive-order-combating-antibiotic-resistant-bacteria
12. The White House. *National Action Plan for Combating Antibiotic-resistant Bacteria* (2015). Available from: https://www.whitehouse.gov/sites/default/files/docs/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf
13. Casagrande R. Biological terrorism targeted at agriculture: the threat to US national security. *Nonprolif Rev.* (2000) **7**:98–9. doi:10.1080/10736700008436827
14. Stadler K, Masignani V, Eickmann M, Becker S, Abrignani S, Klenk H, et al. SARS – beginning to understand a new virus. *Nat Rev Microbio.* (2003) **1**:209–18. doi:10.1038/nrmicro775
15. World Health Organization. *International Standards 2005*. Available from: http://www.who.int/ihr/publications/9789241596664/en/
16. World Organization for Animal Health. *International Standards 1995*. Available from: http://www.oie.int/international-standard-setting/overview
17. Food and Agriculture Organization of the United Nations. *Codex Alimentarius International Food Standards*. Available from: http://www.codexalimentarius.org/standards/en
18. De Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. A pandemic warning? *Nature* (1997) **389**:554. doi:10.1038/39221
19. Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* (2004) **430**:209–13. doi:10.1038/nature02746
20. Exotic diseases close to home. *Lancet* (1999) **354**:1221. doi:10.1016/S0140-6736(99)00177-4
21. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. *Science* (2005) **310**:676–9. doi:10.1126/science.1118391
22. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *PNAS* (2005) **102**:14040–5. doi:10.1073/pnas.0506735102
23. Trifonov V, Khiabanian H, Rabadan R. Geographic dependence, surveillance, and origins of the 2009 influenza A (H1N1) virus. *N Engl J Med*. (2009) **361**:115–9. doi:10.1056/NEJMp0904572
24. Corman VM, Ithete NL, Richards LR, Schoeman MC, Preiser W, Drosten C, et al. Rooting the phylogenetic tree of middle East respiratory syndrome coronavirus by characterization of a conspecific virus from an African bat. *J Virol* (2014) **88**:11297–303. doi:10.1128/JVI.01498-14
25. Hemida MG, Perera RA, Wang P, Alhammadi MA, Siu LY, Li M, et al. Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. *Euro Surveill* (2013) **18**:50. doi:10.2807/1560-7917.ES2013.18.50.20659
26. Han J, Jin M, Zhang P, Liu J, Wang L, Wen D, et al. Epidemiological link between exposure to poultry and all influenza A (H7N9) confirmed cases in Huzhou City, China, March to May 2013. *Euro Surveill.* (2013) **18**:20481.
27. The American Veterinary Medical Association One Health Initiative Task Force. *One Health: A New Professional Imperative* (2008). Available from: https://www.avma.org/KB/Resources/Reports/Documents/onehealth_final.pdf
28. One Health Initiative. Available from: http://onehealthinitiative.com/mission.php

Disclaimer: The views presented in this article represent those of the authors and do not necessarily reflect the position of the the Department of Defense.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

METHODS
published: 15 February 2016
doi: 10.3389/fpubh.2016.00009

Scientific Collaborations: How Do We Measure the Return on Relationships?

Jeanne M. Fair[1], Martha Mangum Stokes[1], Deana Pennington[2] and Ian H. Mendenhall[3]*

[1] Cooperative Biological Engagement Program, Defense Threat Reduction Agency, Fort Belvoir, VA, USA, [2] Department of Geological Sciences, University of Texas at El Paso, El Paso, TX, USA, [3] Duke-NUS Graduate Medical School, Singapore, Singapore

Emerging infectious diseases (EIDs), the majority of which are zoonotic, represent a tremendous challenge for public health and biosurveillance infrastructure across the globe. Due to the complexity of zoonotic pathogens, it is essential that research and response to EIDs be a transdisciplinary effort. And while crisis and circumstance may be the initial catalyst for responding to an outbreak, we provide examples of how transdisciplinary scientific collectives, which are organized and solidified in advance of crises, can transform the way the world responds to outbreaks and in some cases could even prevent one from occurring (1). Current methods for assessing whether a cooperative engagement between countries is producing measurable and sustainable value is based on the ideas of return on investment and do not consider the inherent importance of relationships. In this article, we apply the idea of return on relationships (ROR) and propose a method for measuring ROR, using a system dynamics modeling framework commonly used in epidemiology. Tracking the numerous and diverse scientific collaborations that emerged from a training workshop for biosurveillance of bats held in Singapore in 2014, we apply a methodology for visualizing and measuring the relationship networks and outcomes that result. Additionally, the collaborative, multidisciplinary network that coalesced in response to the Hantavirus outbreak in New Mexico is 1993 is discussed as an example of the long-term benefits of ROR.

Keywords: scientific collaboration, return on relationships, Hantavirus, MERS, systems dynamics

OPEN ACCESS

Edited by:
Hillary H. Carter, U.S. Department of State, USA

Reviewed by:
Paras Jain, Albert Einstein College of Medicine, USA
Peter Smallwood, University of Richmond, USA

****Correspondence:***
Jeanne M. Fair
jeanne.m.fair.civ@mail.mil

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: *23 June 2015*
Accepted: *18 January 2016*
Published: *15 February 2016*

Citation:
Fair JM, Stokes MM, Pennington D and Mendenhall IH (2016) Scientific Collaborations: How Do We Measure the Return on Relationships? Front. Public Health 4:9. doi: 10.3389/fpubh.2016.00009

INTRODUCTION

Emerging infectious diseases (EIDs) represent a tremendous challenge for public health and biosurveillance infrastructure across the globe and are primarily zoonotic (2). Because zoonotic diseases cross species as well as borders, their detection, diagnosis, and prevention require collaboration and communication between a greater diversity of sectors and require national, regional and international coordination (3). A One Health approach (http://www.onehealthinitiative.com) which views the emergence of zoonotic infectious disease as the dynamic interactions of wildlife, domestic animal, human, and environmental ecosystems is required if the various sectors can indeed work together in order to confront these pathogens. Understanding the complex interactions inherent in zoonotic disease requires the union of medical doctors, veterinarians, virologists, wildlife biologists, epidemiologists, and geographers, to name a few. But the very diversity and breadth of a

multidisciplinary team can present challenges to communication and teamwork, particularly in the midst of crisis, when it may be too late to build trust and establish communication mechanisms (1). While there are decades of research on teamwork in a wide variety of settings, few studies consider the dynamics of building informal networks across disciplines and institutions to which result in existing infrastructures of relationships that can be called upon when pathogens emerge and questions need to be answered quickly and collaboratively. A cluster analysis of scientific collaborations in physics by Chompalov et al. (4) revealed that the variety of organizational formats of collaborative projects range from formal and bureaucratic to informal and participatory. Recent studies show that scientific collaboration across disciplines and institutions is growing (5, 6). However, most studies of scientific teams analyze either observed interactions between team members or products of teamwork, especially publications. A better understanding of how collaborations are initiated and sustained through time, and the resulting outcomes (other than publications) is needed. Understanding these processes would enable development of an infrastructure where active surveillance partners can more effectively communicate across EID themes, facilitating responses and interventions.

Both the time scale and the qualitative nature of long-term scientific collaborations pose a challenge to traditional methods of measuring their value and impact. Every event, from trainings, workshops, and conferences to the activities surrounding an outbreak response, requires an investment of both time and monetary resources. And the scientific process necessary to understand the complex systems of zoonotic diseases within the environment requires an investment of years, if not decades, to review what is known, develop ideas and hypotheses, collect and analyze data, and continuously build upon a body of knowledge as new data emerge But assessing the value provided by long-term collaborations, whether those partnerships lead to research outputs, training activities, or simply the sharing of information and best practices, needs to occur within a shorter time scale, if the recognized value to be realized is to inform programmatic and resource allocation decisions. In order to assess the value in that time frame, we need to have a measurement of both the collaborative effort as well as its impact on current disease threat reduction systems. Current indicators that a cooperative engagement or a single event or workshop will produce outputs that are valuable and sustainable are based the measurement of return on investment (ROI) using metrics, such as the number of people trained or number of presentations. In an effort to respond to shrinking scientific budgets, more emphasis is being placed on ROI metrics for applied scientific programs, such as cooperative engagement programs. But the existing ROI metrics, which do not take into consideration the importance of relationships, are not suited for measurement of such intangibles. And how do we demonstrate that investment into the creation and fostering of relationships which are long-lasting, creative, and committed to solving problems can produce truly transformational outcomes which reduce the threat of infectious disease?

The need to develop a framework that captures the impact and benefit from scientific engagements is clear. Policymakers and Program Managers alike need to be able to evaluate when research and training activities are an efficient and effective use of federal dollars, and whether these activities help meet program objectives. But there are challenges to defining the appropriate metrics, including aligning long project timelines with shorter programmatic milestones. Traditionally, the focus has been on reporting research results rather than measuring broader benefits and the synergy of those metrics across different types of activities.

Here, we propose a methodology for visualizing and quantifying the outcomes of such collaborations. We employed the idea of return on relationships (ROR) and propose a method for measuring ROR using a systems dynamics modeling framework. We modify the definition of ROR commonly used in business, as *the long-term net outcome emerging for all parties resulting from the establishment and mutual maintenance of a relational engagement* (7). It is implied that a ROR is an outcome of a mutually reciprocal process and can be assessed on a relationship level, in addition to each member of the relationship (8). Like business, cooperative engagements are reciprocal in nature and may also be appropriate to measure ROR.

Using the scientific collaborations that emerged from the training workshop for biosurveillance of pathogens in bats held in Singapore in 2014, we apply a methodology for visualizing and measuring the resulting relationship networks and outcomes (**Figure 1**). The results of our analysis show that system dynamics principles can be applied to networks of relationships and the resulting information can then be used to measure the impact of efforts and ultimately guide programmatic decision making.

This article analyzes the relationships formed during this single training workshop for sampling wild bats and conducting laboratory analysis. We also discuss a prior analysis of scientific collaborations formed during a disease outbreak and idea of transformative science (1). This prior analysis found that research collaborations with the potential to be transformative depend on human interactions and cooperative foundations within disciplines, effective collaborative mutualism across disciplines, and a communication process that enables knowledge synthesis across diverse perspectives. We demonstrate the evolution of these processes through analysis of workshop participants using a system dynamics model. We show that the outcomes from the workshop can not only be measured but also weighted for relative importance to the team and workshop leaders using a ROR metric. This approach will enhance the efficacy of future collaborative efforts by weighting variables that are not usually considered for success.

MATERIALS AND METHODS

Approach

Similar to bibliometric analysis of scientific co-authorship networks (9), we analyze scientific networks represented through proposals, meetings, posters, and other metrics of scientific output. In contrast to co-author network analysis that represents a series of complex-evolving networks after a period of time has elapsed, our analysis tracks and measures network dynamics during the earliest phase of collaboration. We visualize and model the dynamic and the structural mechanisms that govern

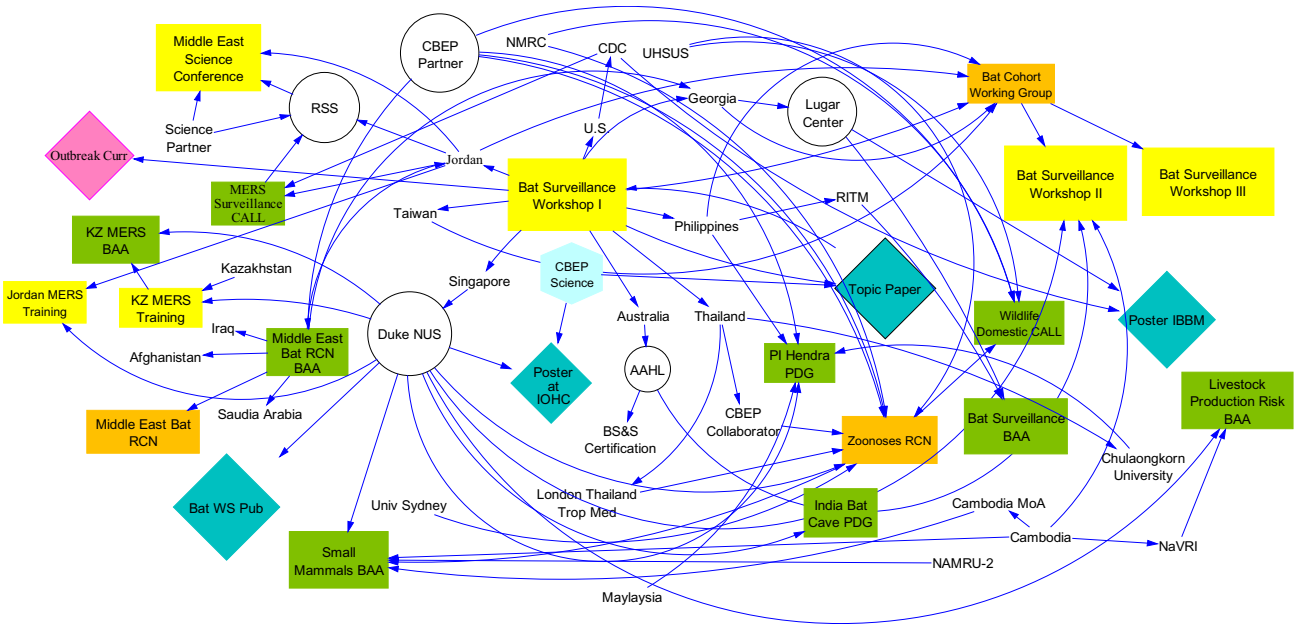

FIGURE 1 | System dynamics model of tracked outcomes of the training workshop (e.g., countries, institutions, and endpoint metrics, such as publications, presentations, and proposals).

the evolution of the complex system of scientific collaborations initiated during a bat-borne pathogen biosurveillance training workshop held in 2014. As an example of the long-term outcomes from an initial group of researchers, we discuss the outcomes from the relationships that developed from the Hantavirus outbreak in New Mexico in 1993. These two events represent the end members of a spectrum of collaborations between scientists. The first event involved deliberate and intentional selection and mixing of potential collaborators, fostering cross-fertilization of ideas across disciplines; the second generated new collaborations due to crisis and circumstance.

The Workshop

In June 2014, members of the Laboratory of Virus Evolution in the Program of Emerging Infectious Diseases at Duke-NUS Medical School in Singapore designed and taught a 2-week course on infectious diseases in bats (Bat Borne Pathogen Surveillance Workshop). Participants were from the Philippines, Taiwan, the Republic of Georgia, Thailand, Jordan, and the USA and were country partners with the U.S.-based Cooperative Biological Engagement Program (CBEP), a component of the Defense Threat Reduction Agency's Cooperative Threat Reduction program. The training, which was initiated and funded by CBEP, leveraged the expertise from the Program in Emerging Infectious Disease at Duke-NUS Medical School, to provide a comprehensive course that covered a wide variety of topics on EIDs in bats. While the primary goal for the bat-borne pathogen training was to increase the capacity and capabilities for biosurveillance in partner countries, a secondary and ultimately perhaps more important goal was to develop a sustainable network for researchers interested in One Health. The participants were able to build on the relationships forged at during the training support and leverage these into research collaborations.

Participants were primarily virologists and laboratory-based scientists, and we provided a course that captured the basics of bat biology, ecology, field surveillance, laboratory screening, and phylogenetic analysis. Thirteen individual lectures were given over the course of 2 weeks. Also included were four field trips to trap and process bats in the field and gain an understanding of the work flow from field to laboratory to analysis (*field to phylogenies*). The final day of the training focused on molecular evolution, sequence alignment, selecting evolutionary models, and phylogenetic tree reconstruction with both lectures and laboratory practicals. All participants were vaccinated for potential zoonotic pathogens, such as rabies, and all animal work was approved by the NUS IACUC (B01/12). A day-long, interactive workshop demonstrated the necessity of a cohesive approach to zoonotic pathogens.

An interactive session was held near the end of the 2-week training and focused on two case studies, the historical Sin Nombre Hantavirus outbreak, which occurred in the Four Corners area of the United States, and the present day Middle East Respiratory Syndrome Coronavirus (MERS CoV) outbreak, which originated in Saudi Arabia but is currently presenting cases worldwide. The goal of the session was to utilize the historic Hantavirus outbreak case study to illustrate how a collaborative approach to problem solving can lead to transformative science. Building upon this example, participants were guided through an exercise where they applied that concept to developing mechanisms (and relationships) to answer questions about the transmission of MERS CoV that remain unanswered today. Finally, the relationships and potential collaborations identified that day and forged during an

intensive 2-week training course in which they lived, worked, and learned together were then tracked and monitored over the next year as the participants returned to their respective countries and institutes.

The eight researchers who came together for this training and workshop represented eight countries in three regions. Like the original, multidisciplinary group of scientists who solved the mystery of Hantavirus transmission in 1993 (1), they brought divergent ideas, scientific cultures, and skillsets together for the purpose of solving one very challenging global health problem. By asking scientific questions across those diverse scientific disciplines and cultures, the potential for unexpected answers can arise. And those answers, as well as the process that generated them, have the potential to drive the creation of new paradigms for scientific problem solving. The transformative science recognized and documented in the discovery of Hantavirus in 1993 (1), which solved a single problem causing a previously unrecognized transmission mechanism, can now be fostered and deliberately directed to aid in solving complex health security issues. But in order to validate this process and demonstrate its effectiveness to scientists, program managers, and policy makers, an analytical metric first had to be developed.

TABLE 1 | System dynamics model weighting for Bat Borne Pathogen Surveillance Workshop I.

Stakeholders	Weighting	Result *N* (weighting)
Countries	2 for country partner 1 for non-partner	14(2) = 28 1
Collaborators, organizations, and institutes	Weighted the same (1)	17
Deliverables (D_i)		
Proposals	1–5 (low to high potential value)	7(5) = 35
Project development grants (PDG)	1–3	2(3) = 6
Publications/posters	1–5	3(3) + 1(5) = 14
Curriculum	1–5	1(4) = 4
Training events/conferences	1–5	5(5) = 25
Capability (assay, standard operating procedures)	1–5	0
Organized meeting with stakeholder and country partners	1–5	10(3) = 30
Event metric ($S + D$): (S) = stakeholders = countries + collaborators; (D) = deliverables = ΣD_i		Total = 114

Weights are on a scale of 1–3 or 1–5 for relative importance or status of deliverable.

System Dynamics of Scientific Collaborations

We developed an aggregate level system dynamics model, which considered the scientific training event (Bat Borne Pathogen Surveillance Workshop) as a resource-limited, finite capacity system operating under control constraints. The scientific social network or "system" that started with the Bat Borne Pathogen Surveillance Workshop were modeled as ordinary differential equations and evaluated in the system dynamics software tool, Vensim™. Mathematically, the basic structure of a system dynamics computer simulation model in Vensim™ is a system of coupled, non-linear, first-order differential (or integral) equations. The Bat Borne Pathogen Surveillance Workshop was modeled as a single starting event that evolved outwards as additional relationships unfolded. The system dynamics model, in this case, is only used to weight the different outcomes that came from a particular relationship and keep track of the endpoints with an overall index. The system dynamics model developed would be considered one of the simplest ways to weight, calculate, and visualize a scientific social network stemming from an event.

Relationships were identified by connections made at the initial training event that led, sometimes by multiple steps or subsequent connections to specific deliverables or outcomes, such as collaboration on a proposal, a paper, or a training event (**Table 1**). Weights were given for deliverables based on a scale of 1–5 for potential low to high value. Scientists or organizations were considered connected if they authored a paper, proposal, or other deliverable together. It is important to note that the weights assigned are based in part on programmatic value and effectiveness in achieving the CBEP goal of providing capabilities to sustain biosurveillance, biosafety, and safe research objectives to strengthen defenses against the threat of infectious disease. Other programs with differing goals and objectives might assign weights differently and even identify other deliverables to track and measure while employing this system of analysis.

Measuring the Return on Relationships

The eight researchers from the Philippines, Taiwan, the Republic of Georgia, Thailand, United States, and Jordan who participated in the workshop each brought unique relationships with institutes, universities, and government offices that could be leveraged for collaborations as deemed appropriate and useful. Each participant had different background and experience in the laboratory and field experience working with animals. We identified a total of 23 measurable deliverables that were the outcomes of the workshop. Nine separate proposals were developed between the participants in the workshop, with smaller project development grants (PDG) being weighted lower than full 3-year proposals for research. Four publications or poster presentations at conferences were developed from the workshop and longer, more developed publications or larger scientific conferences were weighed higher. Even though the original cohort was from seven countries, including Singapore, 12 countries were represented in the outcomes, illustrating the immediate and rapid expansion of the initial collaborative network. One educational curriculum was developed from the workshop, which covered the lessons learned from the Hantavirus outbreak and discovery in 1993 (**Table 1**). Over 20 institutes, universities, or government organizations were brought together through the outcomes from this workshop, and that list continues to grow. Three working groups or research-coordinated networks (RCN) modeled after the National Science Foundation's RCNs in the United States have been formed. The original training curriculum from the Singapore event has been adapted and expanded for use in two other regions where the

participants have begun to tackle the problems and questions presented by the MERS CoV outbreak. Two additional Bat Borne Pathogen Surveillance Workshops with the same cohort have been planned with additional countries being trained in Bat and Camel Pathogen Surveillance, with an emphasis on MERS CoV. For the initial Bat Pathogen Surveillance Workshop, the final value for an index of the number of weighted outcomes and potential relationships that resulted from the workshop was 115 (**Table 1**).

DISCUSSION

The Value of a Single Workshop

A commonly used, numbers-based metric for trainings is the number of people trained or "butts in seats." However, how do we fit the measurement of collaborative efforts in multidisciplinary research and training efforts into a framework of results-based metrics such that the value of ROR can be quantified? How do we demonstrate that investment in the creation and fostering of relationships that are long-lasting and committed to solving problems will result in transformational outcomes that ultimately contribute to reduction of the threat of infectious disease?

Social and collaboration networks are often invisible. That is, they are so interwoven into the fabric of our lives, that they are not observable. As it is often stated, what is not measureable does not exist. As a result, the importance of scientific social networks is often underappreciated and unknown, and consequently undervalued. As with any metric, once scientific social (relationship-based) networks can be identified and measured, emphasis on objectives to increase the metrics can increase. For example, motivation to improve measured results can lead to an increased emphasis in the quality of resulting and differentially weighted deliverables.

For the initial Bat Borne Pathogen Surveillance Workshop, the value of the index or metric was 115, which in the same way as other indices, is not useful without comparison to other events calculated in the same manner. The usefulness of this social network metric lies in the comparison to relevant events to help discern the relative value of the time and resources to organizations. In market-driven business engagements, there are primarily two sides to a value proposition, namely value for the supplier and value for the customer (10). In cooperative engagements, with the shared mission of reducing the threat of diseases, the value in sharing information is realized by improvements in the capability to better detect, diagnose, and report on diseases. With each deliverable, such as a curriculum, manuscript, or training event, communication and trust between the cooperative parties is increased and the value to the shared mission is increased. Within an organization, the benefit for estimating the ROR for an event is in comparing to other events to discern the relative value of often declining, resources. Another benefit for measuring the outcomes from an event is that is can be used in communicating the return of investments to all of the stakeholders in a cooperative engagement. This article demonstrates how a cooperative relationship can be seen as a mutual investment, where reciprocal return on this investment in the relationship can be assessed, and moreover, how this investment pays off as ROR for all stakeholders in the shared mission of reducing the threats of infectious diseases.

Scientific Collaboration and Transformative Science

The One Health initiative explicitly states that interdisciplinary and cross-sectional approaches are required for the prevention, surveillance, monitoring, control, and mitigation of EIDs. The terms multi- and interdisciplinary describe research efforts that incorporate a variety of disciplinary perspectives with varying degrees of integration across perspectives (11). Collaboration between two researchers in different disciplines toward a more comprehensive understanding of a particular infectious disease is an example of multi- or interdisciplinary collaboration. In contrast, transdisciplinary collaboration extends beyond changing our understanding to invoke outcomes beyond the research arena, such as changing the way a disease is diagnosed or treated. Transdisciplinary collaboration explicitly seeks to have transformative outcomes for individuals, professions, or society as a whole, and accomplishes this by intentionally incorporating people and activities designed to convey research results beyond the research community. Hence, the One Health initiative call for interdisciplinary and cross-sectional approaches is an explicit call for transdisciplinary research that has transformative outcomes in all aspects of disease control. And most importantly, the need to support activities designed to foster transdisciplinary collaboration is clear if the collective effort to solve broad global health problems is to succeed.

The Hantavirus Outbreak: An Example

In 1993, a series of unexplained deaths in the American Southwest prompted an immediate response from scientists representing a variety of disciplines and institutions, who ultimately discovered critical linkages between the El Niño Southern Oscillation (ENSO), increased precipitation, vegetation primary productivity, deer mouse (*Peromyscus maniculatus*) populations, and Sin Nombre Virus (12). This transdisciplinary group produced a wealth of outstanding science with immediate, high societal impact.

In a previous analysis of a scientific collaboration that resulted from the Hantavirus outbreak in New Mexico in 1993, it was shown that transformative scientific collaboration depends on human and material foundations within disciplines, effective collaborative mutualism across disciplines, and a learning process that enables knowledge synthesis across diverse perspectives (1). This outbreak prompted new collaborations between clinicians, public health professionals, epidemiologists, pathologists, molecular biologists, and mammalogists and transformed the research of the scientists who were involved, creating new paradigms in the zoonotic infectious disease community and leaving a lasting, positive impact on medical community practices. Specifically, Pennington et al. (1) found that being thrust into disorienting activities associated with rapidly changing information from a variety of disciplinary perspectives initiates transformational learning and postulated that this is a key mechanism for generating new, creative scientific understanding. They argued that

deliberate involvement in transdisciplinary activities in the absence of crisis could potentially result in similar outcomes. This would occur through exposure to unfamiliar concepts, methods, and perspectives that invoke transformational learning and generating new, innovative ideas. Indeed, Pennington (13) found that researchers involved in interdisciplinary workshops were motivated to participate because the exposure to other disciplinary perspectives was having a high impact on their own research and was leading to the generation of creative research ideas (13). Transformational science begins with transforming the perspective of an individual scientist, and this is in direct response to exposure to perspectives besides one's own. A single workshop can have enormous impact if even one potentially transformative idea is generated. This impact is not measurable by any kind of co-author analysis.

In the Hantavirus example, new collaborations formed as a result of the outbreak and a transdisciplinary team was quickly assembled. This was accomplished because the outbreak was local, relevant expertise could be found locally and in the nearby region, and others were able to rapidly converge on the area from elsewhere in the United States. As observed in the recent Ebola outbreak in 2014, global connectivity makes it increasingly likely that infectious outbreaks are not confined to a single locality and easy access to relevant expertise is not assured. A core ideal of CBEPs is one of transparency and collaboration that builds trust between partner countries to facilitate data and information sharing to allow each partner to better respond to infectious disease threats. Transparency and trust are key attributes of successful collaborations that are not easily generated between individuals or organizations (14, 15). Distance effects are very real despite global communications (16). Studies of science teams identified the importance of face-to-face interactions early in a collaborative group (17, 18). These early interactions build transparency and trust and can greatly improve later interactions that are not face-to-face. The Internet has the potential to enhance collaboration among researchers by supporting technical applications that coordinate numerous and complex real-time interactions and can facilitate the rapid dispersal of information between researchers (19) – if transparency and trust are in place (20).

In a study of 699 people working in groups from two to five in a wide variety of settings, Woolley et al. (21) found that the best predictors of creative outcomes were the degree of turn-taking in team interactions and team members' ability to read facial expressions. Intelligence of the team as a whole or of any individual on the team was less important. They proposed a new measure of collective intelligence as a predictor of the creativity of group outcomes (21). A single workshop can begin to generate collective intelligence between participants that is difficult to obtain without the turn-taking and interpretation of facial expressions and body language that occurs in person.

Lessons Learned

The two events differ in that the outbreak response to Hantavirus occurred prior to wide use of the internet and hence, the sustained collaboration that resulted was enhanced by geographic proximity of collaborators. With the ubiquity of the internet modern collaborations are not limited by geography. The Internet has the potential to enhance collaboration among researchers located across the globe by supporting technical applications that coordinate numerous and complex real-time interactions and can facilitate the rapid dispersal of information between researchers (19). Indeed, a core ideal of CBEPs is one of transparency and collaboration that builds trust between partner countries to facilitate data and information sharing to allow each partner to better respond to infectious disease threats.

Using the two examples of the Bat Borne Pathogen Surveillance Workshop and the Hantavirus outbreak response and the resulting networks, what were the lessons learned for increasing the return of relationships? What are things we can do to increase the ROR metric? And are there things that can hurt relationships and break down bridges? From our experience and talking to participants in both events, the following things helped foster stronger relationships between the scientists.

- Having a discussion between all participants on the value of collaborative relationships and the relevance to reducing the threat of infectious diseases helped participants keep a conscious effort for maintaining the collaboration.
- Consistently highlighting and writing down collaborative ideas between participants as they occur resulted in leaving with the event with a collection and outline of collaborative ideas.
- As a last exercise in the event, having participants review the collaborative ideas and develop potential next actions as well as deliverables (metrics) along the way for each party.
- Providing an infrastructure either in funding for additional meetings or an online social site for the group to help strengthen and forge the sustainability of the relationships.
- Finding and giving examples of funding opportunities or supporting scientific collaborations and highlighting deadlines for application.
- Allowing for social activities as part of the event helps foster connections and helping maintain potential future opportunities to meet or connect. Providing opportunities to maintain the relationships over time only builds on the initial connection.
- Not doing any of the above suggestions can impede the success of a research collaborative network, but also forcing participation can backfire. Presenting the data on the positive application of professional networks and continuing to provide opportunities for strengthening the network can invigorate the group to be enthusiastic for working together in the future.

Here, we show that visualizing and quantifying scientific social networks that develop from a specific event can be useful in estimating the impact of collaborations on a field or mission, such as reducing the threat of infectious diseases. A primary goal for cooperative engagement programs is to advance a field together in research or education by supporting groups of investigators to communicate and coordinate their research and training and educational activities across disciplinary, organizational, geographic, and international boundaries. Established relationships may be paramount in preventing the next pandemic.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We would like to thank the Defense Threat Reduction Agency's Cooperative Biological Engagement Program for funding the Bat Pathogen Biosurveillance Workshop. We are grateful to G. Smith and L. Wang for mentorship and guidance on the workshop. We are appreciative of Benjamin P. Y.-H. Lee for his assistance in conducting the workshop. We also wish to thank the staff at Duke-NUS Medical School who volunteered their time to teach, organize, and help with this training. This would not have been feasible without the specific help from Erica Sena Neves, Sophie Borthwick, Dolyce Low, and Udayan Joseph. Our appreciation goes to G. Simpson, M. McConnell, M. Jankowski, M. Baker, and colleagues who provided interesting discussion around these topics at a meeting of the New Mexico Geoepidemiology Research Network. Lastly, the National Science Foundation supported the work that led to the initial analysis of the Hantavirus collaboration, grant #OCI-1135525. Lastly, this work would not have been possible without the enthusiasm, dedication, and feedback from the participants of the Bat Borne Pathogen Surveillance Workshop.

REFERENCES

1. Pennington DD, Simpson GL, McConnell MS, Fair JM, Baker RJ. Transdisciplinary research, transformative learning, and transformative science. *Bioscience* (2013) **63**:564–73. doi:10.1525/bio.2013.63.7.9
2. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. *Nature* (2008) **451**:990–3. doi:10.1038/nature06536
3. Domenech J, Lubroth J, Eddi C, Martin V, Roger F. Regional and international approaches on prevention and control of animal transboundary and emerging diseases. *Ann N Y Acad Sci* (2006) **1081**:90–107. doi:10.1196/annals.1373.010
4. Chompalov I, Genuth J, Shrum W. The organization of scientific collaborations. *Res Pol* (2002) **31**:749–67. doi:10.1016/S0048-7333(01)00145-7
5. Bozeman B, Fay D, Slade CP. Research collaboration in universities and academic entrepreneurship: the-state-of-the-art. *J Technol Transf* (2013) **38**:1–67. doi:10.1007/s10961-012-9281-8
6. Cummings J, Kiesler S. Organization theory and new ways of working in science. *Conference on Science and Innovation Policy*. Atlanta (2011).
7. Gummesson E. Return on relationships (ROR): the value of relationship marketing and CRM in business-to-business contexts. *J Bus Ind Market* (2004) **19**:136–48. doi:10.1108/08858620410524016
8. Gronroos C, Helle P. Return on relationships: conceptual understanding and measurement of mutual gains from relational business engagements. *J Bus Ind Market* (2012) **27**:344–59. doi:10.1108/08858621211236025
9. Barabási AL, Jeong H, Néda Z, Ravasz E, Schubert A, Vicsek T. Evolution of the social network of scientific collaborations. *Phys A Stat Mech Appl* (2002) **311**:590–614. doi:10.1016/S0378-4371(02)00736-7
10. Gupta S, Lehman DR. Customers as assets. *J Interact Market* (2006) **17**:9–24. doi:10.1002/dir.10045
11. Klein JT. Interdisciplinarity and transdisciplinarity: keyword meanings for collaboration science and translational medicine. *J Transl Med Epi* (2014) **2**:1024–30.
12. Yates TL, Mills JN, Parmenter CA, Ksiazek TG, Parmenter RR, Vande Castle JR, et al. The ecology and evolutionary history of an emergent disease: Hantavirus pulmonary syndrome. *Bioscience* (2002) **52**:989–98. doi:10.1641/0006-3568(2002)052
13. Pennington DD. Bridging the disciplinary divide: co-creating research ideas in eScience teams. *Comput Support Coop Work* (2011) **20**:165–96. doi:10.1007/s10606-011-9134-2
14. Stokols D, Misra S, Moser RP, Hall KL, Taylor BK. The ecology of team science – understanding contextual influences on transdisciplinary collaboration. *Am J Prev Med* (2008) **35**:S96–115. doi:10.1016/j.amepre.2008.05.003
15. Cheruvelil KS, Soranno PA, Weathers KC, Hanson PC, Goring SJ, Filstrup CT, et al. Creating and maintaining high-performing collaborative research teams: the importance of diversity and interpersonal skills. *Front Ecol Environ* (2014) **12**:31–8. doi:10.1890/130001
16. Olson GMO. Distance matters. *Hum Comput Interact* (2000) **15**:139–78. doi:10.1207/S15327051HCI1523_4
17. Salas E, Sims DE, Shawn Burke C. Is there a "big five" in teamwork? *Small Group Res* (2005) **36**:555–99. doi:10.1177/1046496405277134
18. Dube L, Robey D. Surviving the paradoxes of virtual teamwork. *Info Syst J* (2009) **19**:3–30. doi:10.1111/j.1365-2575.2008.00313.x
19. Teasley S, Wolinsky S. Scientific collaborations at a distance. *Science* (2001) **292**:2254–5. doi:10.1126/science.1061619
20. Jarvenpaa SLL. Communication and trust in global virtual teams. *Organ Sci* (1999) **10**:791–815. doi:10.1287/orsc.10.6.791
21. Woolley AW, Chabris CF, Pentland A, Hashmi N, Malone TW. Evidence for a collective intelligence factor in the performance of human groups. *Science* (2010) **330**:686–8. doi:10.1126/science.1193147

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on