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Investigating a Mystery Disease: Tales from a Viral Detective

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Viral outbreak investigation is challenging logistically as well as scientifically. In the context of addressing a fictional emerging viral disease, I describe the process of discovery, from the initial report of a problem through discussions of intellectual property and sample management, study design, management, experimental execution, and reporting of results.

Outbreak investigation can be as or more challenging logistically than it is scientifically. This piece is written to provide insights into the entire process, from the initial report of a problem through to resolution, in Charlie Chaplin's fictional country of Tomainia (1). To save on production costs, I will play myself and the Greek chorus. Casting for Dr. X is still open. Enjoy.

> Date: WED 18 NOV 2014 9:00
> From: promed-ahead@promed.org
> Subject: ENCEPHALITIS: TOMAINIA, REQUEST FOR INFORMATION
>
> ProMED-mail has received a report from a reliable source of rumors of the occurrence of an outbreak of fatal encephalitis in children in Tomainia. Further information from any informed person or organization in the area would be appreciated. [C.C.]

SITUATIONAL AWARENESS

Social networks are increasingly important early sources of information on outbreaks. The Program for Monitoring Emerging Diseases (ProMED), founded in 1994, is frequently the first to report an outbreak. Such reports may begin with a request for information or as a report with substantial detail curated by a moderator who provides comments that place the report into context (2).

CONTACT AND VETTING FOR FEASIBILITY

ProMED reports may be rapidly followed by messages delivered by phone or email from clinicians or basic scientists who have access to data and materials but do not have the resources needed for diagnosis and discovery. In my experience, these messages typically come in during the wee hours of the morning. Indeed, I have yet to field an interesting request for assistance during normal business hours. The first step after making contact is to ascertain whether the outbreak is consistent with an infectious disease, whether the usual suspects have been considered, and what type of investigation samples will allow. I have had requests from investigators at reputable institutions to use an oligonucleotide chip to search for evidence of a prion disease. The story was nonetheless intriguing, and we followed it to learn that the outbreak represented an autoimmune disorder triggered by exposure to porcine nervous system tissue (3). A common confounder is the lack of samples suitable for molecular discovery. Paraffin-embedded tissue, for example, may be useful for immunohistochemistry, *in situ* hybridization, or even PCR in cases where assays may have better tolerance for degradation; however, we are reluctant to invest in unbiased high-throughput sequencing (HTS) with materials that have not been stored at −80°C or in buffers designed to preserve nucleic acid integrity.

> Date: November 19, 2014, at 2:39 AM EDT
> From: Dr. X
> To: "W. Ian Lipkin" <wil2001@columbia.edu>
> Subject: Re: hello
> Dear Professor,
>
> There is a challenging problem in northern Tomainia. You may have heard of outbreaks of unexplained encephalitis that annually result in hundreds of deaths of children. I would like to collaborate with you in investigating the causes of these illnesses.
>
> Date: November 19, 2014, at 3:02 AM EDT
> From: "W. Ian Lipkin" <wil2001@columbia.edu>
> To: Dr. X
> Subject: Re: hello
> Dear Dr. X,
>
> We will be happy to collaborate with you on this project. Please suggest a good time to reach you by phone or Skype.

NEGOTIATION

Despite the June 2013 U.S. Supreme Court ruling in the Association for Molecular Pathology vs. Myriad Genetics case that naturally occurring DNA cannot be patented (4), many institutions continue to invest in intellectual property associated with the discovery of microbial gene sequences. It has always been our policy to insist that partners in discovery efforts share equally in whatever equity might arise from collaborations. The financial value of these collaborations is unclear; nonetheless, we continue to include language that reinforces this principle as well academic credit in authorships. A wrinkle that may occur at the publication stage is that authors may be proposed who have had no role in the study design or execution and who may not even have read the

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manuscript. Although it is premature to discuss the criteria for authorship during the initial conversation, it is unwise to leave it until after the manuscript is prepared. Patient confidentiality receives less emphasis in some countries than others. We recommend the deidentification of samples to obviate institutional review board concerns.

Date: November 19, 2014, at 9:18 AM EDT
From: "W. Ian Lipkin" <wil2001@columbia.edu>
To: Dr. X
Subject: Re: hello
Dear Dr. X,

Thanks for taking our call. We will need a material transfer agreement signed by officials at our respective institutions as well as by you and me. As discussed, intellectual property and authorships will be jointly shared. All samples must be deidentified to protect the confidentiality of patients and their families.

MATERIAL TRANSFER, SOVEREIGNTY AND INTERNATIONAL HEALTH REGULATIONS

The Convention on Biological Diversity was initiated by the United Nations Environment Programme in 1998 to codify that biological diversity is a global asset and that benefits arising from the use of such assets (including microbial sequences and strains) should be equitably shared (http://www.cbd.int/convention/). The 2005 International Health Regulations signed by 196 countries across the globe emphasize capacity building as well as transparency and collaborative surveillance. Although a material transfer agreement addressing equity should provide reassurance against exploitation, we increasingly encounter resistance to sample export. Furthermore, samples from ungulates in some countries cannot be readily transported due to the potential risks to agriculture, such as foot-and-mouth disease virus. Accordingly, we have built a mobile laboratory that allows us to pursue nucleic acid extractions, targeted molecular assays, and serology in-country. This laboratory proved invaluable recently in studying the prevalence of Middle East respiratory syndrome coronavirus (MERS-CoV)-infected dromedaries in Saudi Arabia (5). We also train colleagues in the developing world to promote self-sufficiency. Nonetheless, microbial nucleic acid enrichment, library preparation, high-throughput sequencing (HTS), and downstream bioinformatics analysis require state-of-the-art infrastructure. In the 5- to 10-year time frame, therefore, discovery is likely to require a blend of work at the site of the outbreak and in a reference laboratory.

Date: November 20, 2014, at 3:40 AM EDT
From: Dr. X
To: "W. Ian Lipkin" <wil2001@columbia.edu>
Subject: Re: hello
Dear Professor,

Tomainian officials told me that we cannot send human biological material samples from Tomainia abroad. Can we do the work here?

Date: November 20, 2014, at 5:33 AM EDT
From: "W. Ian Lipkin" <wil2001@columbia.edu>
To: Dr. X
Subject: Re: hello
Dear Dr. X,

We will send a team to work with you. However, in the event that PCR fails, I recommend that you join us for a few weeks to finish the work-up with high-throughput sequencing.

THE HUNT BEGINS...

Your colleagues may insist that they have ruled out the usual suspects known to cause disease in a specific catchment area and to ask that you move directly to HTS. Do not do it. More often than not, a few simple consensus PCRs will solve the mystery. Similarly, although our focus here is on virology, keep an open mind to bacterial, fungal, and/or parasitic causes of disease. We have implicated *Plasmodium falciparum* in a medical relief worker who died during a Marburg virus outbreak (6). Additionally, bacteria and viruses may be more pathogenic in concert. During the H1N1 influenza pandemic, the presence of *Streptococcus pneumoniae* in addition to H1N1 influenza virus was associated with a >100-fold increased risk of severe disease (7). We appreciated the importance of coinfection only after sequencing hundreds of influenza viruses in an effort to understand an increase in morbidity and mortality in Argentina.

Communicate with the clinicians as well as the laboratorians. There are exceptions where the application of HTS discovery methods leads to resolution of a single case and a high-profile publication. However, these will typically be cases where one implicates an agent that is known to be pathogenic and a simple solution like targeted consensus PCR would have succeeded more rapidly and at lower cost. Clinicians can be helpful in providing a differential diagnosis that may allow such targeted assays. In an effort to identify clusters of disease, laboratorians frequently oversimplify clinical data. It is important to determine the basis by which clusters have been identified. In the ideal circumstance, cases within clusters are clearly related with respect to timing of onset, geography, subject age, and syndromic features. However, even in the absence of a classical cluster, one can frequently tease out which samples can be considered together and which should not through detailed discussions with the clinicians who collected the samples. Similarly, to avoid investing in samples that may be contaminated or degraded, it is important to communicate with the laboratorian regarding how samples have been processed and stored. Bear in mind the adage "garbage in, garbage out." We have been frustrated more than once to learn in retrospect that gloves or dissecting tools were used continuously to process multiple samples from different individuals, resulting in the appearance of a cluster wherein several individuals were infected with the same agent. Another challenge is laboratories in which sample freezers are adjacent to tissue culture facilities where viruses are passaged. One can sort artifact from reality at later steps in the discovery process through collection of additional samples or testing for the presence of viruses within cells using immunohistochemistry or *in situ* hybridization; however, this is time-consuming and requires additional resources.

CONTROLS

The selection and use of controls in viral discovery projects are critical but complex. In bacterial microbiome projects (I do not think that virologists, mycologists, or parasitologists should cede

the term microbiome to bacteriology), we simultaneously profile bacterial populations of cases and controls based on the assumption that neither cases nor controls are sterile. We typically pursue viral discovery in cases alone. This is not because controls are sterile, but because the lower number of viral sequences in most clinical samples allows one to more easily sift through them to identify candidates that can be tested for relationship with disease by the use of less expensive and more sensitive methods such as PCR. A more critical step is the selection of controls. There are instances where investigators have collected samples from cases of a disease within a specific catchment area during an outbreak of disease and reported identifying the cause of the disease based on the absence of the agent in samples from controls collected during a different season in the same catchment area. It is imperative that controls be selected to ensure that findings in cases are associated with disease rather than variability in virus incidence with season, geography, or socioeconomic status.

FALLABILITY OF THE WILLIE SUTTON RULE

The apocryphal Willie Sutton quote (denied by Sutton) that he robbed banks "because that's where the money is" (8) can direct sampling when the affected organ is readily accessible, as in respiratory, diarrheal, genitourinary, and skin diseases or hemorrhagic fevers. However, central nervous system (CNS) diseases and autoimmune disorders may require a less direct approach. Brain biopsies are rare, and although we have used postmortem samples to enable efficient viral discovery (9, 10), a postmortem diagnosis is not as gratifying as an antemortem finding that impacts patient care. Examples of the latter include the discovery and implication of Lujo virus in a hemorrhagic fever outbreak where the use of ribavirin probably saved one victim (11) and of a novel polyomavirus in a transplant recipient in which assays for viremia facilitated regulation of immunomodulatory therapy, controlling infection while sparing the transplanted organ (12).

Most often, the only available CNS sample is cerebrospinal fluid (CSF). In bacterial CNS infections, CSF culture and PCR are frequently informative; however, with the exception of herpesvirus encephalitis, the rate for resolution of viral CNS infections is 50% or less (13). One can increase this success rate by examining oral and fecal samples by PCR for the presence of viruses known to be associated with CNS infection (e.g., enteroviruses) or by testing CSF for antibodies to neurotropic viruses (e.g., West Nile virus). Some cases of what appear to be infectious encephalitis are in fact autoimmune disorders wherein antibodies bind to brain components such as receptors. Although it is important to distinguish the latter since they respond to immunosuppressive rather than antiviral or antimicrobial therapy, these disorders to not present in clusters, as they typically represent paraneoplastic syndromes. You will also want serum, preferably collected during the illness and a few weeks later, to test for evidence of an immune response to the agent or agents you implicate using molecular methods.

Date: January 14, 2014, at 9:47 AM EDT
From: "W. Ian Lipkin" <wil2001@columbia.edu>
To: Dr. X
Subject: Re: hello
Dear Dr. X,

As discussed, we started with a multiplex PCR assay that targets 20 bacteria and viruses frequently implicated in CNS infections. Five of the 10 children in the July outbreak had enterovirus sequences in their CSF; 9 had enterovirus sequences in their feces. Although we do not have CSF samples from healthy children, only 2 of the 10 outbreak children had viral sequences in their feces. VP1 sequencing indicates that all children were infected with the same genotype. I think we can be confident that this is the agent. Nonetheless, let us build a luciferase immunoprecipitation system (LIPS) assay (14) and test for changes in antibody titer in acute- and convalescent-phase sera. We can also use this same assay to determine the prevalence of infection in the general population and, if you have samples stored from earlier years, when the infection first emerged in Tomainia. We are less clear with the samples from cases collected in the November outbreak: no real clustering was found, just a mix of what appear to be opportunistic infections in association with HIV-cryptococcus, toxoplasma, and Epstein-Barr virus. The December cases are particularly interesting. HTS of brain material from the one postmortem specimen after treatment with nucleases to eliminate nucleic acid not protected within nucleocapsids revealed the presence of a novel flavivirus that we have tentatively named Tomainian flavivirus (TFV). *In situ* hybridization confirms the presence of virus in neurons. We created real-time PCR and LIPS assays and found that 3 of 5 cases had TFV sequences in their CSF and all 5 had antibodies to TFV in CSF and blood. We have also found serum antibodies in 100 of 500 random controls; thus, it seems that while TFV is not a rare infection, the majority of infections do not result in encephalitis. The similarity in presentation to West Nile virus encephalitis led us to investigate mosquito pools using PCR. The vector seems to be *Culex tomainia*. The reservoir is still unknown; however, I have seen reports of dead dodos in Jurassic Park. We could wait to close that loop, but what we have already is more than sufficient for a high-impact publication.

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