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Review

Virus hunting

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ABSTRACT

Viral diagnosis and discovery are receiving increasing emphasis with the recognition of their importance in addressing the challenges of emerging infectious and chronic diseases, and the advent of antiviral drugs with which to reduce the morbidity and mortality of viral infections. Here we review the status of the field including the use of molecular, proteomic and immunological assays for viral detection, social media platforms for surveillance, and public health investments that may enable enhanced situational awareness and insights into the origins of zoonotic viral diseases.

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Introduction

Two quotes from 1960 Nobel Laureate Peter Medawar are particularly apt in introducing this paper on virus hunting: “A virus is piece of bad news wrapped in protein” and “The human mind treats a new idea the same way the body treats a strange protein; it rejects it.” The first is more often cited by clinicians and virologists; however, the latter has proven to be more reliable. Although viruses were once detected only in association with tissue pathology, the advent of molecular methods, and specifically high-throughput sequencing, has enabled the discovery of useful viruses. The retroviral envelope gene syncitin, for example, is critical to placental morphogenesis and may have contributed to mammalian evolution (Mi et al., 2000). Marine phages continuously lyse prokaryotes thereby releasing cellular components that are essential to the growth of phytoplankton, regulation of the global carbon cycle and may play roles in global climate change (Suttle, 2007; Danovaro et al., 2011). Viruses and viral gene products have also been harnessed in biotechnology and medicine. In this paper we will focus on viral discovery, diagnostics and surveillance in acute diseases; nonetheless, we ask the reader, in the spirit of Medawar's second quote, to consider the currently controversial concepts that viruses contribute to chronic disease as

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well as the potential role of viruses as tools for synthetic biology and medicine.

Diagnostics

Viral diagnosis was not a prominent service function in clinical microbiology laboratories until recently. This reflected resource intensive aspects of viral diagnostics such as culture using several cell lines, electron microscopy or serology as well as the fact that results provided insights with minimal impact of clinical management. The status of viral diagnostics has changed with the introduction of antiviral drugs and the development of molecular assays that can identify which patients will benefit from specific antiviral drugs, as well as monitor the adequacy of the responses to those drugs. Additionally, as demonstrated during the course of outbreaks of SARS in 2003, MERS in 2012 and Ebola in 2014, public health practitioners and policy makers are increasingly reliant on viral diagnostics to track emerging viral diseases and make decisions concerning who to isolate and for how long to do so.

Despite the shift in diagnostics toward molecular assays, viral culture continues to play an important role in virology because it is essential to test drugs, the neutralization capacity of antibodies and vaccine responses as well as to develop stocks of virus for work in animal models. Some viruses can be propagated in immortalized cell lines whereas others can only be grown in primary or organotypical cultures. Still others require the use of antibodies or RNAi to suppress innate immune responses or must be inoculated into live animals such as suckling mice. Gastrointestinal samples can be particularly challenging in that they typically contain more than one virus. We have seen many examples where a virus better adapted to culture may outgrow a virus that is more abundant in vivo, obscuring detection of the latter. Accordingly, we prefer to use less biased molecular methods for discovery and reserve culture for follow on studies.

Molecular assays

Molecular assays employed in clinical microbiology include polymerase chain reaction (PCR), isothermal amplification, DNA microarrays, in situ hybridization and sequencing. The most common are real time PCR assays wherein the release of a fluorescent molecule during the course of DNA strand replication results in detection of a single viral target. These assays are exquisitely sensitive, specific, quantitative and inexpensive, and are used not only for differential diagnosis but also to follow response to antiviral therapy. Portable PCR systems have been developed for field applications; nonetheless, some investigators prefer isothermal amplification tests that do not require programmable thermal cyclers. PCR sensitivity is highest when primers and probe sequences perfectly match the selected single genetic target. Indeed, these assays may fail to detect related viruses—a potentially daunting challenge in RNA virus infections where high mutation rates are characteristic. Consensus PCR assays wherein primers and/or probes contain wobble codes may succeed; however, they are typically less sensitive than specific PCR assays. Nested PCR tests that can employ consensus or specific primers in two sequential amplification reactions with either one (hemi-nested) or two (fully nested) primers located 3′ with respect to the first primer set may accommodate sequence variation and be as sensitive as fluorescent real time PCR assays. However, whereas in real time assays reporter readings are taken indirectly without opening the reaction vessels, nested PCR systems are prone to contamination because of the transfer of amplified product from the first to the second nested reaction.

Multiplex PCR assays are increasing in popularity because they can be used to simultaneously address a wide range of candidate viral, bacterial, fungal and parasitic pathogens. This is particularly important early in the course of infection when signs and symptoms of disease are less specific. However, multiplex assays can be difficult to establish because different primers and probes require different reaction conditions for optimal performance. An additional challenge is the limited repertoire of five fluorescent reporter molecules available for use in the most commonly employed clinical instruments. One alternative platform (Luminex xTag) employs flow cytometry to detect multiple PCR amplification products bound to matching oligonucleotides that are attached to fluorescent beads. By combining multiplex PCR amplification systems with various protocols for direct or indirect (tag-mediated) bead hybridization of the products, assay panels have been developed that detect more than 20 different genetic targets. Other PCR platforms employ mass spectroscopy to differentiate genetic targets based on product mass (IRIDICA, Abbott) or the presence of tags conjugated to primers used in PCR that vary in mass (Briese et al., 2005). Such systems can detect up to 20 different genetic targets in a single reaction but are typically 10–100 fold less sensitive than real time PCR.

Microarrays comprising millions of discrete oligonucleotide probes have the potential to detect all known viruses as well as viruses with limited homology to known viruses (Wang et al., 2002; Palacios et al., 2007); however, as they currently depend on a random (vs. specific) PCR amplification step followed by hybridization of the fluorescently labeled product, microarrays are even less sensitive than multiplex PCR systems. New platforms are in development that will detect viral sequence binding through changes in electrical conductance. These platforms will not require fluorescent scanners and may have improved sensitivity.

In situ hybridization is a method whereby nucleic acid probes are used to detect the presence and cellular distribution of complementary sequences in cultured cells or tissue sections. Its primary applications are for work focused on pathogenesis or cell biology rather than diagnostics.

High-throughput sequencing, also known as Next Generation Sequencing, has transformed medicine and virology by enabling viral discovery as well as diagnostics. Unlike PCR or array methods where the breadth of agents interrogated is limited by the capacity for multiplexing or known sequence data, high-throughput sequencing has the potential to simultaneously detect not only all viruses, but also bacteria, fungi and parasites. Furthermore, the time and resources previously required to clone and sequence entire viral genomes have been reduced from months to days. Over the past 10 years the cost has decreased 10,000 fold from $5000 per 1000 nucleotides in 2001 to $0.5 per 1000 nucleotides in 2012.

Platforms in current use analyze libraries of amplified nucleic acids. However, some platforms in development will have the capacity to directly sequence nucleic acid. Irrespective of the platform, raw sequence reads are filtered for quality and redundancy before assembly into contiguous strings of sequence streams that are aligned to sequences in databases using algorithms that search for similarity at the nucleotide and deduced amino acid levels in all six potential reading frames. The alignments allow identification of known and novel agents, as well as detection of genetic features that may be associated with drug or vaccine resistance, or provide insight into provenance and evolution.

Host response

Molecular diagnostic methods are sensitive, but do not serve in instances where microbial nucleic acids are not present in an accessible sample (e.g. the brain in encephalitis) or disease is triggered by an agent that is no longer present. Serology complements molecular diagnostics by providing insights into a subject's pathogen exposure history. It can test for the relevance of a discovery made using molecular methods by indicating that whether an

infection temporally correlates with the onset of disease. Serology can also help target molecular investigation. This application has become less important since the advent of inexpensive unbiased high-throughput sequencing; however, immunohistochemistry and serology were critical in focusing PCR studies that resulted in the identification of Sin Nombre virus (Nichol et al., 1993), Nipah virus (Chua et al., 1999), and West Nile virus in New York City in 1999 (Briese et al., 1999). Although a wide range of diagnostic platforms have been developed for detection of microbial nucleic acids, methods for detection of host responses to pathogens have lagged.

Serological assays provide evidence that a host has encountered and responded to a microbe through the activation of specific B-cells. An activated B-cell can respond to femtomolar antigen concentrations and generate up to 10^9 specific antibody molecules in a week; hence, antibody assays have potential for extraordinary sensitivity. Antibodies are also remarkably stable. To date most serology has been performed using single antigenic target enzyme-linked immunosorbent assays (ELISA). More recently, bead-based systems have been employed that can address up to 100 antigenic targets simultaneously (i.e., 100 individual pathogens, 100 individual antigenic targets for one pathogen, or some variation thereof). Additionally, arrays are established that comprise spotted recombinant proteins expressed in vitro in *Escherichia coli*, Saccharomyces *cerevesiae*, baculoviruses, or in cell-free, coupled transcription-translation systems. However, assay development is complex and time consuming; hence, rapid response to an emerging pathogen is impractical. Furthermore, none of these methods achieve the degree of multiplexing required to complement what has become standard in direct nucleic acid detection systems.

Burbelo et al. (2010) have established a sensitive and quantitative liquid phase format assay for profiling antibody responses to antigen panels. Luciferase immunoprecipitation system (LIPS) assays express viral open reading frames fused to an enzyme reporter, Renilla luciferase, in eukaryotic cells. Fusion proteins harvested under native conditions are mixed with serum samples and immunoprecipitated using protein A/G magnetic beads. No secondary antibodies are required because the primary antibodies in serum bind directly to beads through interaction of their Fc region with staphylococcal protein A and streptococcal protein G. Protein A binds with high affinity to the Fc region of human (IgG1, IgG2, IgM, IgA, IgE), rabbit, guinea pig, dog and monkey immunoglobulins. Protein G binds with high affinity to the Fc region of human, rabbit, pig, horse, cow, mouse, goat and monkey immunoglobulins. Thus, a single LIPS assay can be used to screen many different species for evidence of exposure to infectious agents or for successful vaccination. Establishment of a new LIPS assay typically requires 10–15 days from the time that genomic sequence data becomes available. Over the past two years, we and our colleagues have designed and implemented LIPS assays for investigation of the pathogenesis and epidemiology of astroviruses, picornaviruses, parvoviruses, orbiviruses, hepaciviruses, pegiviruses, and to profile exposure histories of individuals with HIV/AIDS.

Although LIPS technology enables rapid development of serological assays for virology, it is not a multiplex platform. One solution that can address this gap is phage display systems wherein libraries of peptides are reacted with sera and those that bind are characterized through DNA sequencing. Another potential solution is programmable peptide chip technology, an approach analogous to that used in building DNA microarrrays but substituting oligopeptides for oligonucleotides. Commercial platforms are in development that comprise millions of oligopeptide features in a single assay. Such platforms have the potential to enable detection of humoral immune responses to all known vertebrate viruses, allowing surveys for exposure to all known human viral pathogens as well as those that emerge through zoonotic transmission. Through microfluidics it may become feasible to probe an individual's immunological memory, providing insights that will facilitate differential diagnosis and research into the role of specific infectious agents in acute and chronic diseases.

In clinical microbiology, serology is typically interpreted to mean the study of B-cell responses to infectious agents. However, it can be taken in a larger sense to represent any biomarker for infection that is present in serum. The most common approaches are bead-based assays for detection and quantitation of cytokines and chemokines that indicate immune activation consistent with infection. The initial hope was that patterns of expression would be sufficiently distinctive as to allow differential diagnosis by serving as surrogate assays for infection with specific infectious agents. This has not yet proven true; nonetheless, cytokine profiles have been used to for insights into pathogenesis and prognosis (e.g., cytokine storm), indications of genetic differences underlying difference in innate and adaptive immunity and as biomarkers for viral reactivation.

Even more robust than the cytokine platforms currently limited to analysis of 50–70 molecules are the biomarker discovery platforms that quantitate levels of specific host RNA populations in tissues and body fluids through use of microarrays or RNA sequencing (transcriptomics), host proteins (proteomics) or products of metabolic processes (metabolomics) through mass spectroscopy. Together these assays comprise the components of the field of systems biology wherein investigators integrate large datasets for insights into the impact of the environmental perturbations on function at the level of individual cells, organs or the entire organism. These insights have the potential to lead to treatment strategies that modify host response rather than targeting the infectious agent. They can also be used to identify genetic and epigenetic determinants of host response that influence the outcome of infection. Recently, such research in genetically characterized mice infected with Ebola virus provided clues that may help explain differences in human vulnerability to this hemorrhagic fever virus (Rasmussen et al., 2014).

Viral discovery and surveillance

Viruses represent the largest and most genetically diverse source of biomass on earth (Suttle, 2005). Suttle has estimated that the ocean alone contains more than 10^{30} viruses, with an abundance that exceeds that of archea and bacteria by at least 15-fold. Characterization of the global virome is clearly an insurmountable task; fortunately, our focus here is on the only slightly less daunting challenge of characterizing the repertoire of human viral pathogens.

Zoonotic diseases and globalization

The vast majority of emerging infectious diseases, including such examples as HIV/AIDS, Nipah, SARS, MERS and Ebola, represent infections with viruses that jump species barriers from wildlife or domestic animals to humans. Although the role of animals in emerging infectious diseases has been known for decades, it is only recently that it was formally recognized by the establishment of the One Health Initiative (http://www.onehealthinitiative.com/), a movement to enhance collaboration between health care professionals, public health practitioners and policy makers. The emergence of zoonotic diseases can be attributed to behaviors that result in increased frequency of human exposure to wildlife through incursion into wildlife habitats or consumption of wildlife products (bushmeat) as well as conditions that facilitate human-to-human transmission of infectious agents such as high population density and mass gatherings. A warming climate may also increase the

geographic range of phlebotomous insects like mosquitoes and ticks that serve as reservoirs and vectors for infectious agents.

Globalization of travel and trade is also an important factor in the emergence and dissemination of viral diseases. Nonstop flights of less than 24 h connect the world's major airports. Legal and illegal trafficking of animal products and exotic pets can result in inadvertent importation of exotic infectious diseases once restricted to the developing world. The annual traffic in bushmeat through JFK airport alone in New York is estimated at more than 90 million tons. Our analysis of bushmeat seized at JFK revealed evidence of infection with retroviruses and herpesviruses (Smith et al., 2012). Illegal importation of birds, primates and rodents has been linked to outbreaks of exotic viral diseases. The most prominent example was in 2003 when 35 people were infected with monkeypox carried from Africa by a Gambian pouched rat, brought in through the illegal pet trade (Di Giulio and Eckburg, 2004).

Several programs have been established with the goal of proactively addressing the challenge of infectious disease emergence through targeted surveillance in global 'hotspots' of emergence. The largest is the Emerging Pandemics Threat Program (EPT), administered by the United State Agency for International Development (USAID). The goals of the EPT, and of programs sponsored by other governments and foundations are to understand the underlying causes of disease emergence and spread in people through enhanced local wildlife surveillance, and the delivery of new platforms for surveillance and discovery close to the source of pathogen emergence.

Sampling: how much is enough?

A question that arises frequently in discussions of viral surveillance and discovery is how much sampling would be required to obtain a comprehensive inventory. As a case study, we recently addressed this question by repeatedly sampling a mammalian wildlife host known to harbor emerging zoonotic pathogens (the Indian Flying Fox, *Pteropus giganteus*), and used PCR with degenerate viral family-level primers to discover and analyze the occurrence patterns of viruses representing nine viral families. Asymptotic viral richness, estimated by adapting statistical techniques more commonly used to estimate biodiversity in vertebrates and plants, indicated that *P. giganteus* likely contained at least 58 viruses in these nine families. A simple extrapolation to all 5486 described mammalian species then indicated that a minimum of 320,000 viruses await discovery. This finding then allowed us to consider an economic analysis to estimate the global cost of discovering these viruses, or some portion of them. With rapidly diminishing returns in discovery (as a function of effort), we demonstrated that 85% of the estimated diversity could be achieved for a fraction of the cost of discovering them all, highlighting the potential usefulness of this approach in refining targeted public health surveillance.

Bioterrorism and synthetic threats

Naturally emerging hemorrhagic fevers and respiratory diseases are the primary foci in viral biosurveillance for public health; nonetheless, bioterrorism concerns also drive policy. Until the close of the Cold War in the late 1980s, the major concern was bioweapons research at the State Research Center of Virology and Biotechnology VECTOR in Novosibirsk. Concern thereafter shifted to the Middle East and accelerated after the twin towers aerial attack in 2001 and the subsequent anthrax attacks. One result of these events in the U.S was the establishment of new BSL-3 and BSL-4 laboratories and the NIAID Regional Centers for Biodefense. These enabled the recruitment of scientists to develop diagnostics, drugs and vaccines for high-threat pathogens. An early objective of the centers was to address the challenge of genetically modified pathogens; however, as building such pathogens required resources and expertise that were then present in only a few highly specialized laboratories in the industrialized world, this concern rapidly receded to allow a focus on emerging infectious diseases. In the intervening years, synthetic biology has become more straightforward and there is again a need to include surveillance for high-threat engineered pathogens. These may be the products of bioweapon development by rogue scientists or of legitimate gain-of-function research with inadvertent release.

Pathogenesis and proof of causation

As more investigators become involved in the field of pathogen discovery, it is becoming increasingly important to develop criteria for establishing a causative relationship between the presence of a virus and a disease. The best-known criteria for proof of causation are known as Koch's postulates. These criteria require that an agent be present in every case of the disease, be specific for the disease and sufficient to reproduce the disease after culture and inoculation into a naive host. Koch's postulates remain the ideal; nonetheless, there are instances where they cannot be fulfilled because there is no culture system for propagating the virus or animal model for testing pathogenicity.

We have developed a hierarchy of confidence in the relevance of a viral discovery (Fig. 1) (Lipkin, 2013). Level 1, a *possible causal relationship*, pertains when there is evidence of exposure to an infectious agent in one or more individuals with disease. This evidence might be a virus recovered in culture or visualized by electron microscopy, or a viral nucleic acid or protein. Level 2, a *probable causal relationship*, pertains when the link is biologically plausible because evidence of infection is found in many individuals with the same disease, there is precedent for a similar disease caused by a similar virus in either the same or a similar host, the virus (or its gene products) are found at high concentration at the site of pathology, or there is a host response consistent with recent exposure (virus-specific IgM or rise in virus-specific IgG). Level 3, a *confirmed causal relationship*, requires fulfillment of Koch's postulates or evidence that the use of specific drugs, antibodies or vaccines can prevent or mitigate disease.

Host factors can have a profound impact on susceptibility to infection and the consequences of infection. Agents that normally cause no or only mild disease can have severe consequences in individuals with immunodeficiencies, due to genetic mutations, age, malnutrition, HIV/AIDS or complications of cancer treatment or transplantation. Thus, the principle whereby investigators look for clusters of disease associated with candidate pathogens must be expanded from simple models where cases are closely linked in time and space to include clusters based on other demographic factors. A classic example is the association between progressive multifocal leuokoencephalopathy due to JC virus and immunosuppression in the context of HIV/AIDS or therapy for multiple sclerosis. Pathogenic mechanisms may be direct or indirect, readily manifest or subtle. Viruses can cause tissue damage as a direct result of replication or via innate or adaptive immune responses to microbial gene products. Microorganisms can also induce neoplasia through interference with cell cycle controls. Although not yet confirmed in human disease, work in animal models indicates that viruses can reduce the production of hormones or neurotransmitters that are vital to normal host physiology, and that they can do so without causing any apparent cell or organ damage.

Some of the most intriguing concepts in viral pathogenesis involve complex mechanisms that include infection with other microbes (other viruses, bacteria or parasites) or a timing component. The most straightforward instances are those where viral infection results in immunosuppression that enables opportunistic infections. The most dramatic example is HIV/AIDS where disease

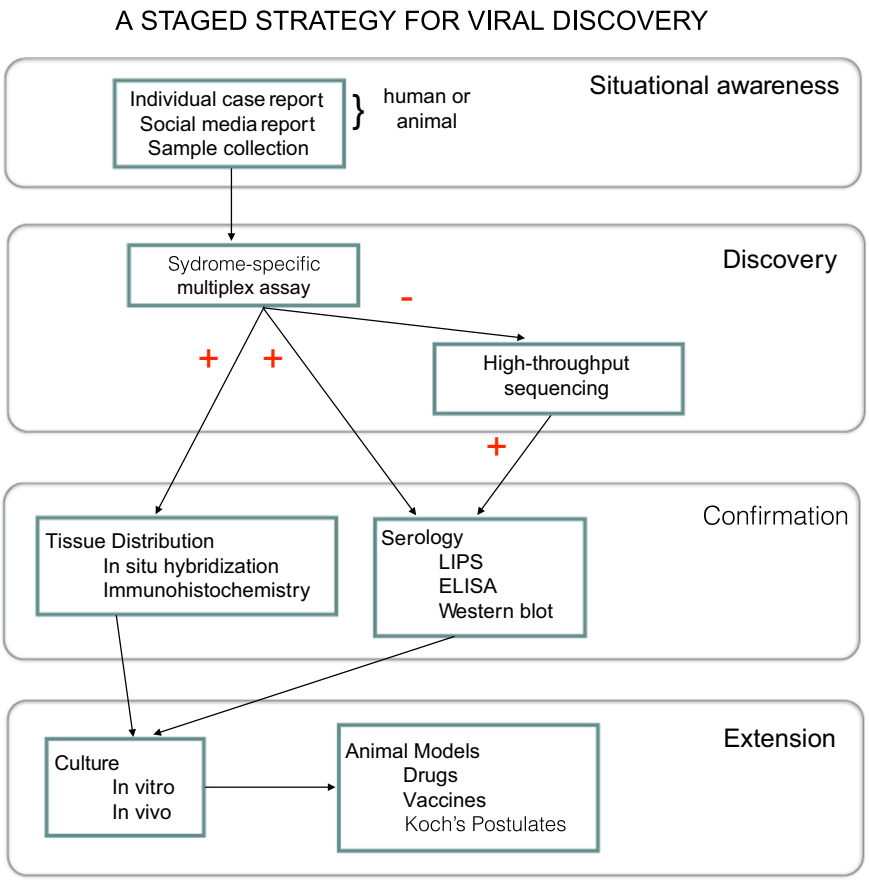

Fig. 1. A staged strategy for viral discovery. The process initiates with the report of illness consistent with a case or outbreak of infectious disease and collection of appropriate samples (tissue, blood, cerebrospinal fluid, urine, feces or respiratory secretions). In the discovery phase, samples are analyzed using a syndrome-specific molecular assay, most commonly based on the polymerase chain reaction. If the multiplex assay yields a candidate viral sequence, the process advances to the confirmation phase. If not, samples are subjected to unbiased high-throughput sequence analysis to identify potential pathogen candidates. In the confirmation phase, the relationship of the virus to disease is tested by examining the site of viral replication and determining whether disease is associated with an adaptive immune response to the virus. In the extension phase, efforts are made to propagate the virus in cultured cells or animals to enable research in cell biology and pathogenesis, and to develop interventional strategies including antiviral therapies and vaccines.

may present with *Pneumocystis carinii* pneumonia, central nervous system infection with *Toxoplasma gondii* or *Cryptococcus neoformans*, or Kaposi sarcoma. A related phenomenon is expansion of the enteric virome in Rhesus macaques infected with SIV (Handley et al., 2012). Bacterial superinfection has been implicated in increased morbidity and mortality in influenza infection (Wang et al., 2002; Palacios et al., 2009). Helminth infection in mice can result in reactivation of gamma herpesviruses through induction of IL4 (Reese et al., 2014).

We and others have built models of neurodevelopmental disorders based on gestational viral infections wherein the timing of maternal inoculation with influenza virus or the viral mimic polyinosine/cytosine results in abnormalities in social and locomotor behaviors in the offspring (Malkova et al., 2012). These effects are associated with a reduction in neuronal stem cells that is found in wildtype mice but not in Toll 3 receptor knockout mice and can be abrogated through the use of nonsteroidal anti-inflammatory drugs (De Miranda et al., 2010).

Our ability to detect associations between environmental exposures and disorders that may only manifest in later life depends on access to prospectively collected questionnaire data and clinical samples in large cohorts. The Center for Infection and Immunity works with the Norwegian Institute of Public Health in a program that includes such questionnaire data and samples obtained beginning at the first prenatal visit (approximately 17 weeks gestation). The cohort represents 117,000 mothers and their children, and is linked to a national patient registry that records all clinic and hospital visits (Stoltenberg et al., 2010). In reviewing such registries, investigators have an unprecedented resource with which to examine how infection, intoxication and genetic and epigenetic factors influence risk and resilience for diseases in any organ system.

Social media

The development of the Internet and of web protocols that facilitate exchange of user-generated content offers an unprecedented opportunity for global biosurveillance. The first such program was ProMED-mail (Programme for Monitoring Emerging Infectious Diseases) (www.promedmail.org). Since 1994, ProMED-mail has continuously collected submissions that report confirmed or suspected new or evolving outbreaks and epidemics. Submissions are screened and commented upon by a panel of experts, then distributed in several languages to a listserv comprising more than 60,000 subscribers in 185 countries. GPHIN (Global Public Health Intelligence Network) established in collaboration with the World Health Organization Global Alert Response Network scans global media for information concerning disease outbreaks, food and water contamination, bioterrorism, natural disasters, and reports concerned with the safety of products, drugs and medical devices (Mykhalovskiy and Weir, 2006). Unlike ProMED-mail, which is a freely available, GPHIN is a fee-based subscription service. HealthMap collects reports from professional and social media including crowd sourced information contributed via its website or smart phone-based apps such as Outbreaks Near Me and

Google Flu Trends. Data are then assembled into a user-friendly graphical interface that provides real-time updates of disease, with hyperlinks to more detailed information (http://healthmap.org/en/). There is as yet no comprehensive system that collects information concerning medical service utilization, death records, diagnostic data, and prescription and over-the-counter drug use. However, the future of biosurveillance will almost certainly include all of the above.

Modeling viral emergence

Improvements in diagnostics and discovery methods and the early detection of outbreaks using social media hold the promise of reducing the mortality, morbidity and health care costs due to emerging viral diseases. Better still would be a proactive strategy in which surveillance and discovery efforts are focused at sites and in hosts wherein viral pathogens of humans are likely to emerge and re-emerge. The development of high resolution, global scale data sets of human demographics, agricultural production, land-use change, travel and trade patterns, climate and wildlife distribution has provided tools that may ultimately enable accurate predictive modeling; nonetheless, it should be acknowledged that to date no viral human outbreak has been predicted using algorithms prior to a cross-species jump. These authors have not ruled out the possibility that such algorithms will be developed but believe that our best defense against emerging viral diseases is to develop local capacity in laboratory diagnostics and outbreak response as well as a robust global surveillance system wherein data and samples are shared to promote public health.

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