Microbe Hunting in Laboratory Animal Research

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Abstract

Recent advances in nucleic acid diagnostic technologies have revolutionized microbiology by facilitating rapid, sensitive pathogen surveillance and differential diagnosis of infectious diseases. With the expansion and dissemination of genomic sequencing technology scientists are discovering new microbes at an accelerating pace. In this article we review recent progress in the field of pathogen surveillance and discovery with a specific focus on applications in the field of laboratory animal research. We discuss the challenges in proving a causal relationship between the presence of a candidate organism and disease. We also discuss the strengths and limitations of various assay platforms and describe a staged strategy for viral diagnostics. To illustrate the complexity of pursuing pathogen discovery research, we include examples from our own work that are intended to provide insights into the process that led to the selection of particular strategies.

Key Words: high-throughput sequencing; MassTag; microbe; molecular assay; pathogen discovery; pathogen surveillance; polymerase chain reaction (PCR)

Introduction

Increasingly globalized travel and trade, climate change and its effects in the distribution of phlebotomous arthropod vectors, and shifts in demographics and land use have ushered in an era of infectious disease outbreaks and pandemics (Morse 1995). The advent of potent threats to health and economic welfare—human immunodeficiency virus (HIV), severe acute respiratory syndrome (SARS[1]), H1N1 (swine influenza), and H5N1 (avian influenza)—has created unprecedented support for basic and translational research in host, vector, and microbe biology as well as diagnostics, surveillance, vaccines, and therapeutics. New molecular platforms such as MassTag polymerase chain reaction (PCR[1]) (Briese et al. 2005; Lamson et al. 2006; Palacios et al. 2006; Renwick et al. 2007), microbial microarrays (Chiu et al. 2006; Palacios et al. 2007; Wang et al. 2002), and unbiased high-throughput pyrosequencing (Cox-Foster et al. 2007) have enabled efficient differential diagnosis, pathogen discovery, and surveillance in clinical infectious disease. In addition, enhanced surveillance of wildlife, domesticated animals, and other known vectors (e.g., rodents, mosquitoes), especially in "hot spots" of infectious disease emergence, has better prepared the public health, medical, and research communities to predict and avert the next pandemic (Deem et al. 2001; Jones et al. 2008; Wolfe et al. 2005).

Viral Discovery in Laboratory Animal Research

Health surveillance of laboratory animals is essential because adventitious infection is a continuous challenge to animal welfare and research. Rodent parvoviruses, *Helicobacter* spp., murine norovirus, and other previously unknown infectious agents have recently "emerged" in laboratory rodents. These agents have been discovered serendipitously or through active investigation of atypical serology results, cell culture contamination, unexpected histopathology, or previously unrecognized clinical disease syndromes. Although a daunting and expensive proposition, most facilities can establish clean colonies through strict quarantine and breeding policies and, where needed to preserve critical transgenic lines, embryo transfers (Smith 2010).

A different scenario pertains in work with nonhuman primates. Given longer generation times, domestic production cannot meet current or projected research needs for rhesus (*Macaca mulatta*) or cynomolgus (*M. fascicularis*) macaques, so imported animals will play an increasingly critical role into the foreseeable future. While the two types of macaques are available for importation from Southeast Asia and Mauritius, because of a lack of national and international standards for microbial quality control for research animals, these animals represent an undefined and uncharacterized population with regard to infectious and parasitic agents. The significance of the problem is underscored by recent outbreaks of tuberculosis, measles, and simian malaria and the detection of antibiotic-resistant *Shigella flexneri* in recently imported rhesus of Chinese origin, and the well-publicized 1989 outbreak of

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[1]Abbreviations used in this article: cPCR, consensus PCR; PCR, polymerase chain reaction; RT-PCR, real-time PCR; SARS, severe acute respiratory syndrome

hemorrhagic disease and subsequent recognition of the previously unknown Ebola-Reston virus in cynomolgus macaques imported from the Philippines (Jahrling et al. 1990). Because it has not been possible to eliminate all pathogens from nonhuman primate colonies, it is important to define the spectrum of microbial agents present in a population of animals and understand how these agents influence pathobiology and experimental work (Mansfield and Kemnitz 2008).

Infectious and parasitic diseases may adversely affect research programs in several ways (Lerche and Osborn 2003). Many agents are well-documented pathogens, causing overt morbidity and mortality. Unrecognized or adventitious agents are common in nonhuman primates and have the potential to confound experimental studies. Nonpathogenic, commensal agents in normal host species may represent a zoonotic disease risk for human contacts (e.g., *Macacine herpesvirus* 1) or become pathogenic in normal hosts secondary to experimental manipulations or immunosuppression. The presence of undetected microbial agents—pathogens and nonpathogens alike—represents a potential threat to the health status of breeding colonies, human contacts, and research integrity. Thus one of the biggest challenges in research that uses imported nonhuman primates is the need for improved diagnostic tools to screen for infectious and parasitic agents (Roberts and Andrews 2008; Simmons 2008).

Proof of Causation

Indicating the presence of a microbial organism is only the first step in elucidating its relationship to disease. In acute infectious disease the agent can often be isolated, morphological changes correlate with the distribution of the agent, and serology provides evidence of an adaptive immune response. However, not all agents can be cultured and clinical signs and symptoms of infection are not specific to particular organisms. Furthermore, factors such as the host's genetic susceptibility, age, nutrition, and previous exposure to similar agents may modulate expression of disease.

Implication of agents may be difficult when classical hallmarks of infection are absent or mechanisms of pathogenesis are indirect or subtle. In these cases investigators must resort to a statistical assessment of the strength of epidemiological association based on (1) the presence of the agent or its footprints (nucleic acid, antigen, and preferably an immune response) and (2) the biological plausibility indicated by analogy to diseases with related organisms where linkage is persuasive. Such evidence may be sufficient to merit interventional trials. Examples include hepatitis B and papillomaviruses, human infections for which vaccination prevents infection with the candidate organisms and reduces the incidence of hepatic or cervical neoplasia, respectively.

Strategies for Pathogen Discovery

Reviews such as this typically focus on the latest molecular technologies, but clinical acumen, pathology, serology, and classical culture techniques still play a pivotal role in pathogen discovery. Only clinicians and epidemiologists can appreciate the appearance of anomalies, collect materials for investigation, and persuade their laboratory colleagues to invest in the search for known and novel pathogens.

Immunohistochemistry can be instrumental in directing molecular work by exploiting the cross-reactive properties of antisera to reveal the presence of agents related to the original immunogen. The potency of this approach is underscored by the fact that the discoveries of Sin Nombre virus (Chizhikov et al. 1995), Nipah virus (Paton et al. 1999), West Nile virus (WNV; Briese et al. 1999; Lanciotti et al. 1999; Shieh et al. 2000; Steele et al. 2000), and LuJo virus (Briese et al. 2009) were facilitated by demonstration of viral proteins in tissues, which in turn allowed focused consensus PCR (cPCR[1]) analyses.

Classical methods such as tissue culture and serology are also important. Tissue culture was pivotal in the 2003 SARS outbreak (Peiris et al. 2003), wherein growth of the virus enabled rapid characterization by using cPCR, random primed cDNA libraries, microarrays, and electron microscopy.

Since the advent of PCR, methods for cloning microbial nucleic acids directly from clinical specimens have become commonplace in pathogen surveillance and discovery. Over the past 2 decades, subtractive cloning, expression cloning, cPCR, and high-throughput pyrosequencing resulted in identification of the following novel agents (in addition to the LuJo, Nipah, and Sin Nombre viruses) associated with both acute and chronic diseases: Borna disease virus, hepatitis C virus, human herpesvirus (HHV) 6, HHV-8, *Bartonella henselae*, *Tropheryma whipplii,* SARS coronavirus, and Israel acute paralysis virus (Briese et al. 2009; Challoner et al. 1995; Chang et al. 1994; Choo et al. 1989; Cox-Foster et al. 2007; Lipkin et al. 1990; Nichol et al. 1993; Paton et al. 1999; Peiris et al. 2003; Relman et al. 1990, 1992; VandeWoude et al. 1990).

Singleplex Assays

The most sensitive molecular assays are those in which primers and probes have perfect complementarity to a single genetic target. Examples include fluorescence reporter–based TaqMan or molecular beacon singleplex PCR assays that may have detection thresholds as low as 5 RNA molecules and are ideal for applications focused on detecting the presence of a specific agent or quantitating viral burden (Heid et al. 1996; Tyagi and Kramer 1996). Yet these assays can fail with viruses characterized by high mutation rates and genetic variability. Degenerate primers and probes can be designed to accommodate sequence divergence, although this typically entails a compromise in sensitivity. Perhaps the most significant confound is that signs and symptoms of disease are rarely agent-specific, particularly early in the clinical course; thus, unless an investigator has sufficient sample, resources, and time to invest in many singleplex assays for

different agents, there is the risk that the wrong candidate(s) will be selected among the many potential pathogens that can overlap in clinical presentation.

The most common singleplex assays used in clinical microbiology and microbial surveillance are PCR assays that enable the detection of fluorescent signal as DNA strand replication results in either cleavage or release of a labeled oligonucleotide probe bound to sequence between the forward and reverse primer. Equipment needs are modest (a thermal cycler, fluorescence reader, and laptop computer), so reliable fluorescent reporter–dye singleplex assays are achievable under field conditions, with battery power if necessary.

Loop-mediated isothermal amplification (LAMP) is an alternative to PCR that does not require programmable thermal cyclers (Hagiwara et al. 2007; Notomi et al. 2000; Shirato et al. 2007). Although its sensitivity is reported to be similar to that of PCR, LAMP is not quantitative. Products are typically detected in ethidium bromide–stained agarose gels, but changes in turbidity of the amplification solution may be sufficient; indeed, according to at least one study, the accumulation of product was detectable by eye (Jayawardena et al. 2007).

Nested PCR, where two amplification reactions are pursued sequentially with either one (hemi-nested) or two (fully nested) primers located 3' with respect to the original primer set, may be more sensitive than fluorescent reporter–dye singleplex assays; however, the potential for contamination is higher because the original reactions must be opened to add reagents for the second, nested, reaction (Casas et al. 1997; Templeton et al. 2004). Nested PCR is challenging even in laboratories with scrupulous experimental hygiene.

Degenerate Systems of Amplification

Representational difference analysis (RDA) is an important tool for pathogen identification and discovery. However, it is a subtractive cloning method for binary comparisons of nucleic acid populations (Hubank et al. 1994; Lisitsyn et al. 1993). Thus, although ideal for analysis of cloned cells or tissue samples that differ in only a single variable of interest, RDA is less well suited to investigation of infections (with any of several different pathogens) that result in similar clinical manifestations or that are not invariably associated with disease. An additional caveat is that because the method requires the presence of a limited number of restriction sites, RDA is most likely to succeed for agents with large genomes—indeed, the two viruses detected by RDA were herpesviruses (Challoner et al. 1995; Chang et al. 1994).

Consensus PCR has also been a remarkably productive tool for biology (Strand et al. 2000). In addition to identifying pathogens, this method has facilitated identification of a wide variety of host molecules, including cytokines, ion channels, and receptors. But it also presents limitations; for example, for use in pathogen discovery in virology, it is difficult to identify conserved viral sequences of sufficient length to allow cross hybridization, amplification, and discrimination in a traditional cPCR format. Although this may not be problematic when one is targeting only a single virus genus, the number of assays required becomes infeasible when preliminary data are insufficient to permit a more directed, efficient analysis.

To address this problem, we adapted cPCR to differential display, a PCR-based method for simultaneously displaying the genetic composition of multiple sample populations in acrylamide gels (Liang and Pardee 1992). This hybrid method, known as domain-specific differential display, employs short, degenerate primer sets designed to hybridize to viral genes that represent larger taxonomic categories than can be resolved in cPCR. While this modification allowed us to identify WNV as the causative agent of the 1999 New York City encephalitis outbreak (Briese et al. 1999), it did not resolve issues of low throughput with cPCR due to limitations in multiplexing.

Multiplex PCR Assays

Multiplex assays allow the investigation of many hypotheses simultaneously. At the same time, they are more difficult to establish than singleplex assays because each individual target primer set needs to share the same optimal reaction conditions (e.g., annealing temperature, magnesium concentration). Moreover, complex primer mixtures are more prone to primer-primer interactions, which reduce assay sensitivity and/or specificity. Specific software tools are available to automate consensus primer design over a multiple sequence alignment and allow users to specify primer length, melting temperature, and degree of degeneracy compatible with the multiplex PCR approach (Jabado et al. 2006).

Gel-based multiplex assays, wherein products are distinguished by size, can detect as many as 8 to 10 distinct targets, albeit with low sensitivity (Casas et al. 1997; Templeton et al. 2004). These designs are yet more cumbersome than others to set up since they add the additional constraint that PCR products need to be resolved adequately by electrophoresis. Fluorescence reporter–based multiplex assays are more sensitive but limited by the number—four—of fluorescent emission peaks that can be unequivocally separated. "Sloppy molecular beacons" can circumvent this limitation in part by binding to related targets at different melting temperatures (Saunders and Jeffries 2000); however, as they cannot detect targets that differ by more than a few nucleotides, the improvement does not approach the multiplex capacity of gel-based assays.

Two multiplex platforms join PCR and mass spectroscopy (MS) for sensitive detection of several targets. One of them, triangulation identification for genetic evaluation of risks (TIGER), uses matrix-assisted laser desorption/ionization (MALDI) MS to directly measure the molecular weights of PCR products obtained in an experimental sample and to compare them with a database of known or predicted product weights (Hofstadler et al. 2005; Sampath et al. 2007; Van Ert et al. 2004). TIGER uses cPCR to detect viruses of a

given species or genus and then compare their signature with a database of known agents. It has the potential to directly indicate candidates for novel variants of known organisms via a divergent product mass but it requires sequencing to characterize a novel sequence or agent. MALDI-MS (TIGER) is confined to specialized laboratories, and the possibilities for multiplexing are limited to detection of members the same families, genus, or species.

The other system, MassTag PCR, uses atmospheric pressure chemical ionization (APCI) MS to read molecular weight reporter tags attached to PCR primers (Briese et al. 2005; Palacios et al. 2006). APCI-MS (MassTag PCR) can be performed on smaller, less expensive, portable instruments. Syndrome-specific MassTag PCR panels enable rapid differentiation of viruses, bacteria, fungi, and parasites associated with acute respiratory disease, diarrhea, pustular diseases, encephalitis/meningitis, tick-borne associated infectious agents, and hemorrhagic fevers (Briese et al. 2005; Lamson et al. 2006; Palacios et al. 2006; Tokarz et al. 2009).

Other multiplex PCR systems use flow cytometry to detect amplification products bound to matching oligonucleotides on fluorescent beads (Brunstein and Thomas 2006; Han et al. 2006; Li et al. 2007). Sensitivity is similar across these platforms. Whether any will become dominant in the commercial arena remains to be seen.

Viral Microarrays

Viral microarrays can be broadly divided into those that address 10 to 100 agents and those designed for detection of thousands of agents including unknowns. Arrays in the second category (e.g., respiratory virus resequencing arrays) may use multiplex cPCR to amplify specific genetic targets (Chiu et al. 2006, 2007; Miro et al. 2007; Wong et al. 2004). Sample preparation is not complex and sensitivity in clinical materials is typically 10^3 to 10^4 RNA copies. Although these arrays, which typically use probes less than 25 nucleotides (nt), may allow speciation of agents, they do not truly exploit the utility of microarrays for unbiased microbe detection.

Oligonucleotide microarrays can comprise hundreds of thousands of features. Probes of up to 70 nt are not uncommon; thus, unlike PCR or resequencing arrays, where short primer sequences demand precise complementarity between probe and target, such arrays are less likely to be confounded by minor sequence mismatches. Additionally, one can incorporate both microbial and host gene targets in high-density arrays. This affords an opportunity to both detect microbes and assess host responses for signatures consistent with various classes of infectious agents.

The two most familiar larger scope platforms are the Virochip and the GreeneChip (Palacios et al. 2007; Wang et al. 2003). They differ in methods used for probe design, array manufacture, hybridization conditions, and bioinformatics analysis of hybridization results; what is common to both is that sample nucleic acids are randomly amplified and labeled. This unbiased amplification is critical to exploiting the full range of probes representing tens of thousands of viral targets. However, because host and microbe sequences compete with similar efficiency for PCR reagents, sensitivity for microbial detection is commonly low (10^6 to 10^7 copies). It is not surprising, therefore, that the most successful applications of this technology have been in assays of clinical samples with only low host nucleic acid content (e.g., serum, cerebrospinal fluid, or urine). Improvements in sensitivity (10^3 to 10^4 copies) have been achieved with more challenging tissue samples by depleting host cell DNA and ribosomal RNA (rRNA) before amplification and labeling, and by using as reporters labeled dendrimers that bind to hybridized sequences to enhance overall signal intensity.

In some array platforms hybridization is detected as changes in electrical conductance. These have not yet extended to high-density microarrays but have the potential to further enable improvements in sensitivity. Viral arrays can facilitate cloning and sequence analysis as well as pathogen identification. Hybridized products typically range from 200 nt to more than 1000 nt. Because arrays display probes representing several different genomic regions for each virus, one can rapidly recover sequence not only for the hybridized products but also for sequences between those products through use of PCR. The method is simple: the hybridized products are eluted with hot water and reamplified using the specific sequence portion of the primers originally used for random amplification.

High-Throughput Pyrosequencing

High-throughout sequencing affords unique opportunities for pathogen discovery. Unlike cPCR or array methods, where investigators are limited by known sequence information and must make choices about the range of pathogens to consider in a given experiment, high-throughput sequencing is unbiased and makes it possible to consider the entire tree of life: bacteria, viruses, fungi, and parasites. Our experience is primarily with the pyrosequencing system of 454 Life Sciences, but the principles for sample preparation and data analysis are broadly applicable across platforms (Margulies et al. 2005).

As in viral microarray analyses, elimination of host nucleic acid is critical to boosting pathogen signal toward the threshold for detection. Although this approach reduces the potential for detecting DNA genomes of pathogens, our reasoning is that an active infection should be associated with transcription. After amplification and sequencing, reads typically range in size from 40 to 400 base pairs. Raw sequence reads are trimmed to remove sequences derived from the amplification primer and filtered to eliminate highly repetitive sequences. After trimming and eliminating repeats, sequences are clustered into nonredundant sequence sets. Unique sequence reads are assembled into contiguous sequences, which are then compared to the nonredundant sequence databases using programs that examine homology at the nucleotide and amino acid levels using all six potential reading frames (Palacios et al. 2008).

A Staged Strategy for Pathogen Detection and Discovery

We view the platforms described above as complementary tools. Due to ease of use, sensitivity, capacity for quantitation, and low cost, singleplex PCR is ideal in instances where the questions relate to the presence of a single agent or viral burden. Examples include (1) outbreaks of acute infectious disease where decisions concern patient containment or allocation of specific interventions that are in short supply or potentially toxic, or (2) adjustments of antiviral regimens as in HIV infection.

Multiplex assays are indicated when singleplex PCR fails to identify an agent, or the clinical presentation and context are not pathognomonic of infection with a single agent. The most time- and cost-effective alternatives for such second-stage analyses are multiplex PCR platforms. Where they fail or the list of candidate agents exceeds 25 to 30, microarrays are indicated. Multiplex PCR and microarray platforms both require that an agent be related to one already known. In instances where agents are novel or sufficiently distant in sequence to related agents to confound hybridization it may be necessary to pursue subtractive cloning or unbiased high-throughput sequencing.

Irrespective of the method that results in identification of a pathogen candidate, subsequent steps include quantitation of pathogen burden in affected hosts and controls, detailed characterization of the pathogen for features that may contribute to virulence or provide clues to provenance, and serology as an index to acute infection and a tool to examine prevalence of infection over time and geography.

Although unbiased high-throughput sequencing is becoming an increasingly popular tool for pathogen discovery as costs decrease, it may fail where the agent is present at low levels or the sequence is so different that commonly used algorithms do not detect it as microbial.

Subtractive cloning may succeed where unbiased high-throughput sequencing fails by boosting nonhost signal. Indeed, we use it to enhance the efficiency of unbiased high-throughput sequencing platforms. Borna disease virus, the first agent discovered with unbiased molecular methods (Lipkin et al. 1990), would not have been found by consensus PCR, microarray, or high-throughput sequencing because its genomic sequence is so dissimilar to other agents.

New analytical models based in neurolinguistics and cryptography may facilitate recognition of microbial sequences even when they lack similarity to known microbes at the primary sequence level.

Vignettes in Pathogen Discovery

Borna Disease Virus and Neuropsychiatric Disease

The story of the molecular characterization of Borna disease virus (BDV) illustrates how far the field of pathogen discovery has evolved over the past 20 years. Until the mid-1980s, BDV was known as an unclassified infectious agent named after a town in Saxony (Eastern Germany) that had large outbreaks of equine encephalitis in the late 1800s. In 1985, Rott and Koprowski reported that serum from patients with bipolar disorder reacted with BDV-infected cells (Rott et al. 1985). The concept that infection might be implicated in a neuropsychiatric disease intrigued us and, challenged by the fact that established methods for virus isolation had failed, we and others began to pursue characterization using only molecular tools.

The isolation of BDV nucleic acids by subtractive hybridization in 1989 was the first successful application of this technique in pathogen discovery (Lipkin et al. 1990). The fact that it was performed relying on home brew kits for cDNA cloning and that it preceded the advent of PCR and ready access to sequencing technologies underscores the challenges microbe hunters faced even a few years ago. Sequencing was not a mainstream technology at the time the first cDNAs were obtained. Furthermore, even if it had been available, researchers would not have appreciated the similarity of BDV to other viruses. The association between cloned materials and disease was achieved by demonstrating that (1) candidate cDNAs competed with RNA template from the brains of infected rats for transcription and translation of a protein biomarker present in the brain (hybrid arrest experiments), (2) the distribution of candidate nucleic acid correlated with pathology in the brains of experimentally infected rats and naturally infected horses (in situ hybridization), and (3) no signal was obtained in Southern hybridization experiments wherein normal brain was probed with candidate clones.

Over the next 5 years the genome was cloned and the virus visualized and classified as the prototype of a new family of nonsegmented negative-strand (NNS) RNA virus with unusual properties: nuclear replication/transcription, post-transcriptional modification of selected mRNA species by splicing, low-level productivity, broad host range, neurotropism, and capacity for persistence (Briese et al. 1992, 1994; Cubitt et al. 1994; de la Torre 1994; Schneemann et al. 1995; Schneider et al. 1994).

Although it was widely believed that the introduction of specific reagents such as recombinant proteins and nucleic acid probes would allow rapid assessment of the role of BDV in human disease, there is still no conclusive evidence that the virus infects humans. In what could be considered a classic example of the pitfalls of PCR diagnostics, particularly using nesting methods, BDV was erroneously implicated in a wide variety of disorders—unipolar depression, bipolar disorder, schizophrenia, chronic fatigue syndrome, AIDS encephalopathy, multiple sclerosis, motor neuron disease, and brain tumors (glioblastoma multiforme) (Lipkin et al. 2001; Schwemmle et al. 1999). Nonetheless, BDV discovery was fundamental to the emergence of intriguing new models of viral pathogenesis; moreover, the research provided invaluable guidance regarding methods to investigate the role of infection in chronic disease with sensitive molecular tools.

West Nile Virus Encephalitis

The introduction of PCR changed the landscape of pathogen discovery. The story of the discovery of West Nile virus in North America is an example of the power of this technology. It was also instrumental in demonstrating the importance of expanding infectious disease surveillance beyond humans to domestic animals and wildlife.

In late August 1999, health officials reported an outbreak of encephalitis accompanied by profound weakness in Queens, New York. Recognition of the syndrome was not due to an apparent increase in the frequency of encephalitis per se, nor an automatic reporting event that resulted in detection of the outbreak, but to the clinical acumen of Deborah Asnis, an infectious diseases physician at Flushing Hospital Medical Center, and Marcelle Layton, Assistant Commissioner of the Communicable Disease Program at the New York City Department of Health, and their associates.

In early September, serology for the presence of antibodies to North American arboviruses yielded results consistent with infection with St. Louis encephalitis virus (SLEV) (Asnis et al. 2000). Although mosquito vectors competent for transmission of SLEV were present, the virus had not been previously reported in the state. Early victims of the outbreak had histories consistent with mosquito exposure; investigation of the outbreak epicenter was consistent with the nearby existence of sites of active mosquito breeding. Thus, on September 3, the state and city of New York implemented a mosquito eradication program.

Concurrently, wildlife observers independently noted increased mortality of avian species including both free-ranging crows and exotic birds housed in the Bronx Zoo. Tracy McNamara, a veterinary pathologist at the Wildlife Conservation Society, performed histologic analysis of the birds and found meningoencephalitis, gross hemorrhage of the brain, splenomegaly, and myocarditis (Steele et al. 2000). Although 70% of emerging infectious diseases are zoonoses and in this instance the coincidence between the human and nonhuman outbreaks was striking, McNamara was unable to persuade her colleagues in human infectious disease surveillance to review materials. She forwarded tissue samples from diseased birds to the US Department of Agriculture (USDA) National Veterinary Service Laboratory in Ames, Iowa, where a virus was cultured and electron micrographs were consistent with the presence of either a togavirus or a flavivirus. Only then was the avian virus forwarded from USDA to the Centers for Disease Control and Prevention (CDC) Division of Vector-Borne Infectious Diseases in Fort Collins for molecular analysis (Lanciotti et al. 1999).

On September 13-15, the CDC Encephalitis Project (comprising centers in California, New York, and Tennessee) held its annual meeting in Albany, New York. Data from both California and New York over an 18-month survey period indicated that despite culture, serology, and molecular analyses, 70% of cases of viral encephalitis remained without an etiological agent. Sherif Zaki at CDC Atlanta had demonstrated the presence of flavivirus protein in the brains of human victims of the New York City outbreak, but efforts to amplify SLEV or other flaviviral sequences by conventional real-time (RT) PCR[1] had been unsuccessful.

Our group was invited to discuss methods for identification of unknown pathogens and to consider their application to project samples. Using several degenerate primer sets designed to target highly conserved domains in the NS3, NS5, and 3' untranslated regions of flaviviruses, we obtained positive results for four of the five New York patients in only a few hours. Sequence analysis confirmed the presence of a lineage 1 WNV (Briese et al. 1999; Jia et al. 1999). Concurrently, our colleagues at the CDC in Fort Collins reported West Nile–like sequences in cell lines infected with homogenates from New York birds (Lanciotti et al. 1999). Both findings confirmed that the outbreak in New York City was a zoonosis due to West Nile virus.

Subsequently, we established quantitative RT-PCR assays for sensitive high-throughput detection of the virus in clinical materials and mosquito pools. Although analysis of blood samples from infected humans revealed the presence of WNV sequences in late 1999 (Briese et al. 2000), the significance of human-human transmission was not appreciated until 2002, when transmission through organ transplants and blood transfusion led to the implementation of blood screening by nucleic acid amplification tests (CDC 2003, 2004).

This outbreak underscores the significance of enhancing communication between the human and comparative medicine communities.

Human Rhinovirus C

During the 2004-2005 influenza season, the New York State Department of Health received 166 samples through the CDC Influenza Surveillance System. Samples were analyzed using antigen detection and RT-PCR assays designed to identify influenza viruses as well as conventional virus culture for the detection of additional respiratory viral pathogens. These analyses identified a candidate agent in 48% of samples (some negative samples were collected more than 10 days after onset of symptoms, when low microbial load at the time of collection could have accounted for assay failures). However, many of the negative samples clustered during October to December of 2004, an observation compatible with the circulation of an unidentified agent during that interval.

We pursued this hypothesis using a MassTag PCR panel that targeted influenza viruses A and B; human respiratory syncytial viruses A and B; human coronaviruses OC43, 229E, and SARS; human parainfluenza viruses 1 through 3; human metapneumovirus; human enterovirus (HEV); human adenovirus; and *Mycoplasma pneumoniae, Legionella pneumophila, Chlamydia pneumoniae, Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria meningitidis*. MassTag PCR identified a pathogen in 26 (33%) of the previously negative specimens (Lamson et al. 2006). The degenerate HEV primers used in the MassTag PCR assay amplify conserved regions in the 5' untranslated region (UTR) of picornaviruses that are

also found in human rhinoviruses (HRV). When samples that had tested positive with this primer pair were tested with a specific diagnostic RT-PCR assay for HEV, 17 of the 18 cases yielded a negative result. Reasoning that products represented either novel HEV or HRV isolates, we cloned all MassTag PCR amplification products. Sequence analysis identified 2 HEVs and 16 HRVs. More detailed phylogenetic analyses of other gene regions indicated that eight cases represented a novel rhinovirus clade distinct from the group A or B serotypes. Follow-up studies in Europe, Australia, and Asia confirmed an international distribution of these viruses and revealed that this novel genetic clade plays a major role in pediatric asthma and pneumonia (Kistler et al. 2007; Lau et al. 2007; McErlean et al. 2007; Renwick et al. 2007). At the time of writing there are no reports that a representative of this novel clade has been in grown in culture.

These studies confirm the importance of novel multiplex molecular methods for surveillance, outbreak detection, and epidemiology.

Dandenong Virus

In December 2006 three patients in Melbourne, Australia, received solid organ transplants on the same day from a single donor (Palacios et al. 2008). The donor was reported to be in good health until he died of a hemorrhagic stroke approximately 10 days after returning to Australia after a 3-month trip through Southeastern Europe. The three organ recipients died 3 to 4 weeks after transplantation after a clinical course marked by fever and encephalopathy. Extensive pre- and postmortem workups with bacterial and viral cultures as well as PCR for a wide range of bacterial and viral pathogens were unininformative. When MassTag PCR and GreeneChip microarray assays of RNA from the recipient organs, plasma, and cerebrospinal fluid yielded no evidence of infection, the same RNA was subjected to unbiased high-throughput pyrosequencing, yielding a total of more than 100,000 nucleotide sequences. Using bioinformatic algorithms, human sequences were subtracted, and nucleotide and deduced amino acid sequences were compared with genetic databases to identify related microbial sequences. Whereas S-segment sequence was recognizable at the nucleotide level, footprints of the L-segment were detected only at the amino acid level, consistent with a previously unknown Old World arenavirus. Specific PCR analyses confirmed the presence of the same virus in all recipients. Tissue homogenates from organs with the highest viral RNA titer were used to inoculate cell culture lines. Infected cells were used to develop an indirect immunofluorescence assay for serology and to obtain electron micrographs of the agent.

LuJo Virus

On September 1, 2009, a woman with recent onset of severe headache, fever, chest pain, and pharyngitis was admitted to a hospital in Lusaka, Zambia. She rapidly deteriorated and was transferred by air to Johannesburg, South Africa, for specialized care. During the flight a physician and a paramedic attended to her with pulmonary toilet and manual ventilation. On arrival in Johannesburg she was comatose with cerebral edema, acute respiratory distress syndrome, and renal failure. She died shortly thereafter. No autopsy was performed, and the body was returned to Zambia for cremation. The physician and the paramedic who attended her during the air evacuation, a nurse who cared for her in Johannesburg, and a custodian responsible for cleaning hospital rooms developed a similar clinical syndrome with fatal outcomes.

When initial workup of clinical materials failed to identify a causative agent for this outbreak, the World Health Organization coordinated a multicenter diagnostic effort at the National Institute for Communicable Diseases in South Africa, the CDC, and Columbia University. Immunohistochemistry revealed footprints of an arenavirus and cPCR confirmed its presence. Within 72 hours of sample receipt, unbiased high-throughput pyrosequencing yielded nearly complete genomic information of a novel arenavirus that was named LuJo virus (after *Lu*saka and *Jo*hannesberg) (Briese et al. 2009; Paweska et al. 2009). Based on these data, a fifth case of acute illness (a nurse who cared for one of the secondary victims) was identified through contact tracing and treated with the antiviral drug ribavirin. She recovered.

The time course of the LuJo outbreak and the rapidity with which the agent was identified and characterized illustrate the power of the sequencing technologies for public health and personalized medicine.

Future Perspectives

Many of the tools described above are available in only a few specialized laboratories in the industrial world. While it is unlikely that the full complement of technologies will be broadly available in the near future, it is essential to establish the capacity for differential diagnosis of infectious diseases in the developing world, where the risk and burden of hemorrhagic fevers are most prominent. Toward this end we are encouraged that academicians, public health practitioners, and corporate partners are beginning to focus on smaller footprint solution phase and microarray platforms that promise to perform in resource-poor environments. Investment in the surveillance of bush meat, wildlife, domestic animals, and humans in geographic hot spots at increased risk for emerging infectious disease emergence is long overdue. Here, too, we are encouraged by recent increases in support of these proactive efforts.

Detection technologies will continue to evolve, allowing faster, more sensitive, and less expensive methods for pathogen surveillance and discovery. Multiplex PCR assays are already widely implemented, but microarray technology is less advanced. Improvements in development include microfluidic sample processing and direct measurement of

conductance changes associated with hybridization. Although we have not addressed proteomics and host response profiling, investigators may yet discover biomarkers that either are specific for classes of infectious agents or provide insights that can guide clinical management.

Active collaboration between clinicians and laboratorians is key to success in diagnostics. The most advanced technology will fail if samples are collected without attention to nucleic acid lability; data will be uninterpretable without accurate information on clinical course and sample provenance. In chronic diseases, where complex mechanisms such as early exposure and/or genetic susceptibility may contribute to pathogenesis, the most substantive advances in linking microbes to disease are likely to come from investments in prospective serial sample collections and an appreciation that many diseases reflect intersections of genes, species, and environment in a temporal context.

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