Diagnostics and Discovery in Viral Hemorrhagic Fevers

W. Ian Lipkin, Gustavo Palacios, and Thomas Briese

Center for Infection and Immunity, Mailman School of Public Health of Columbia University, New York, New York, USA

The rate of discovery of new microbes and of new associations of microbes with health and disease is accelerating. Many factors contribute to this phenomenon including those that favor the true emergence of new pathogens as well as new technologies and paradigms that enable their detection and characterization. This chapter reviews recent progress in the field of pathogen surveillance and discovery with a focus on viral hemorrhagic fevers.

Key words: **epidemiology; virus; biology**

Introduction

Globalization of travel and trade, changes in demographics and land use, and climate change have ushered in an era wherein viral hemorrhagic fevers and respiratory virus pandemics are no longer obscure.[1] Popular media have focused attention on biodefense and emerging infectious diseases providing a foundation for unprecedented support of basic and translational research in host, vector, and microbe biology, as well as diagnostics, surveillance, vaccines, and therapeutics. New molecular technologies, such as MassTag polymerase chain reaction (PCR),[2–5] microbial microarrays,[6–8] and unbiased high throughput pyrosequencing[9] have facilitated efficient differential diagnosis of infectious diseases, as well as pathogen surveillance and discovery. The databases needed to recognize sequences as host or microbial have improved dramatically. Appreciation that more than 75% of emerging infectious diseases represent zoonoses has also had an impact. To address this issue, sample collection needs to be more proactive in addressing infections of wildlife, domestic animals and vectors as well as humans, examining bush meat, and promoting surveillance in regions defined through modeling as "hot spots" of infectious disease emergence.[10–12]

With the advent of countermeasures tailored to specific viruses, including small molecules, RNAi, therapeutic antibodies and vaccines, accurate early differential diagnosis of viral infection is increasingly important in clinical medicine as well as public health. Treatment decisions must frequently balance potential benefit with potential toxicity from antiviral therapy. When toxicity is minimal, but supplies are limited, it may nonetheless be imperative that a drug be reserved for those cases for which it will be effective. Finally, treatment is frequently more effective early in the course when the viral burden is lower. It is important to appreciate that agents other than viruses can cause hemorrhagic fevers. In one recent example, an individual who succumbed to acute febrile illness and multi-organ failure during an outbreak of Marburg hemorrhagic fever was ultimately found through panmicrobial microarray analysis to have malaria.[8] Effective antimalarial therapy might have been instituted had the appropriate diagnosis been established at an earlier time point.

Addresss for correspondence: W. Ian Lipkin, Center for Infection and Immunity, 722 West 168th Street, 18th Floor, New York, NY 10032. Voice: 212 342 9033; fax: 212 342 9044. wil2001@columbia.edu

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Proof of Causation

Finding footprints of an organism is only the first step in establishing a causal relationship or understanding how it causes disease. Nonetheless, in viral hemorrhagic fevers, continuing the discovery process through to implication of a candidate is typically straightforward because disease is acute, the agent is present at high levels in the affected tissue at the time the disease is manifest, morphological changes consistent with infection are evident, classical or molecular methods for isolation and characterization are commonly successful, and there is frequently evidence of an adaptive immune response. Thus, even where one does not have the animal model required to formally meet the bar for proof of causation established by Koch and Loeffler (the agent is present in every case of a disease; it is specific for that disease; it can be propagated in culture and inoculated into a naïve host to cause the same disease),[13] a strong candidate is rarely controversial.

Methods for Viral Diagnosis, Surveillance, and Discovery

Reviews, such as this one, typically focus on the latest molecular technologies. Nonetheless, it is important to recognize the pivotal roles of clinical acumen, pathology, serology, and classical culture techniques. Clinicians and epidemiologists are the unheralded heroes at the front line in pathogen discovery. They appreciate the appearance of anomalies, collect materials for investigation, and persuade their laboratory colleagues to invest in the search for known and novel pathogens. Anatomic pathology joined to immunohistochemistry can be instrumental in directing molecular work. Here investigators exploit the cross-reactive properties of antisera to reveal the presence of agents related to those already known. Although the large panels of antisera required for this type of work are restricted to a few highly specialized research centers, the potency of this approach is underscored by fact that the discoveries of Sin Nombre virus,[14] Nipah virus,[15] West Nile virus[16–19] and LuJo virus[20] were facilitated by demonstration of viral proteins in tissues, which in turn, allowed focused consensus PCR analyses. Methods, such as tissue culture and serology, are also important. Tissue culture was pivotal in the 2003 SARS outbreak[21] wherein growth of the virus enabled rapid characterization by using consensus PCR, 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 two decades, subtractive cloning, expression cloning, consensus PCR, and high throughput pyrosequencing resulted in identification of novel agents associated with both acute and chronic diseases, including Borna disease virus, Hepatitis C virus, Sin Nombre virus, HHV-6, HHV-8, *Bartonella henselae*, *Tropheryma whippelii*, Nipah virus, SARS coronavirus, Israel Acute Paralysis virus, avian borna virus, and LuJo virus.[9,15,21–29]

In clinical microbiology, singleplex PCR assays are increasingly implemented to detect and quantitate the burden of individual candidate organisms. Such assays have revolutionized blood banking, management of HIV and helpatitis C virus, and enabled containment of outbreaks of infectious disease. Degenerate primers can be employed in singleplex PCR assays at reduced stringency to facilitate detection of related but unknown organisms. However, clinical manifestations are not typically pathognomonic of infection with specific pathogens; thus, unless an investigator has clues from pathology or serology that can be used to focus a search, this is a cumbersome strategy even if sample, resources, and time are sufficient to invest in many singleplex assays for different agents. Multiplex assays, however, allow an investigator to test many hypotheses in parallel. The number of candidates considered in such assays can range from less than

10 with multiplex PCR, to thousands with microarrays, to the entire tree of life with unbiased high throughput sequencing.

A Staged Strategy for Viral Diagnosis, Surveillance, and Discovery

Although in the context of outbreaks where time is of the essence, we pursue several methods simultaneously, in less urgent situations we try to contain costs of pathogen discovery by staging investment. Frequently, epidemiology, serology, or pathology suggests one or a few candidates, allowing the use of consensus PCR with degenerate primers. Where no such clues are known or assays for single agents fail, *syndromic multiplex PCR assays* can be used to test several candidates simultaneously. Gel-based multiplex assays that distinguish products by size continue to be used. However, these assays are more labor intensive and less sensitive than assays based on fluorescence reporter dyes or mass spectrometric analyses, where automation enables high throughput and sensitivity that approaches that of singleplex PCR. Fluorescence reporter-based assays take several forms: those where fluorescence reporter dyes are attached to primers (Scorpion; Premier Biosoft, Palo Alto, CA), others where an intermediate labeled oligonucleotide is cleaved (Taqman; Applied Biosystems Inc., Foster City, CA) or hybridized (molecular beacons) to separate an excitatory and quencher moiety, and another where amplification products bind to labeled beads (Luminex Corporation, Austin, TX). Two multiplex platforms have been developed that combine PCR and mass spectroscopy (MS) for sensitive detection of several targets simultaneously. 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.[30,31] The other, MassTag PCR, uses Atmospheric Pressure Chemical Ionization (APCI) MS to read photocleavable molecular weight reporter tags attached to PCR primers.[2,3,32]

Microarrays may be helpful where appropriate multiplex panels have not been established or if multiplex PCR fails.[6,8,33,34] However, sensitivity is lower than for the multiplex PCR methods. Viral microarrays can be coarsely divided into those that address 10–100 agents and those designed for detection of thousands of agents including unknowns. Arrays designed to address a limited number of agents, for example respiratory virus resequencing arrays, may employ multiplex consensus PCR to amplify specific genetic targets.[33] Although these arrays, which typically employ probes of less than 25 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 variation. This can be a considerable advantage for detection of rapidly evolving targets, such as RNA viruses. 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. Viral arrays can facilitate cloning and sequence analysis as well as pathogen identification. Following random amplification protocols, hybridized products typically range from 200 nt to >1000 nt. Because arrays display probes representing several different genomic regions for each virus, one can rapidly recover sequence by elution, not only for the hybridized products, but also for sequences between those products through use of PCR amplification and classical dideoxy-sequencing.

Sensitivity is a major challenge to array technology that also applies to unbiased

high throughput sequencing. Both rest on an amplification step that amplifies host and microbial sequences with similar efficiency. In acellular samples, such as serum or tissue culture medium, this is not an issue because host nucleic acid is minimal; however, in tissues, host nucleic acid is more likely to be dominant. Chromosomal DNA can be eliminated through DNase digestion; however, ribosomal RNA is more difficult to address. We have seen only modest improvements in sensitivity with rRNA capture or digestion.

Mutliplex PCR methods and, to a lesser extent, microarrays, require that an agent be closely related to one already known. Agents sufficiently distant in sequence to confound hybridization to primers or probes may still be identified by *unbiased high throughput sequencing*.[35–38] Depending on the platform employed, this strategy yields sequence fragments that typically range in size from 30 to 500 nt. These fragments are then used to query databases for similar sequences that represent host or microbe. By definition, truly novel microorganisms will not be represented in a database. However, we have been able to detect similarities of 40% at the aa level that are sufficient for genus level identification that were not detected at the nt level. Even where an agent is identified using other, less expensive and labor intensive methods, unbiased high throughput sequencing may be employed because this approach eliminates time consuming cloning prior to sequencing needed with classical dideoxy methods. An example here is the Bundibugyo virus, a new ebolavirus species associated with a recent hemorrhagic fever outbreak in Uganda, that was discovered using conventional methods, but genetically characterized through pyrosequencing.[39] *Subtractive cloning* may succeed where unbiased high throughput sequencing fails. Borna disease virus, for example, the first agent discovered with unbiased molecular methods, 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 primary sequence level.

Future Perspectives for Viral Diagnosis, Surveillance, and Discovery

Many of the tools described in this chapter 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 distributed in the near future, it is essential that the capacity for differential diagnosis of infectious diseases be established in the developing world where the risk and burden of hemorrhagic fevers is 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 surveillance of bush meat, wildlife, domestic animals, and humans in geographic hot spots that are 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.

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Conflicts of Interest

The authors declare no conflicts of interest.

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