Diagnosis, Discovery and Dissection of Viral Diseases

2

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Only a few years ago, viral diagnosis was largely an exercise for academic researchers and public health practitioners with focus on epidemiologic analyses and outbreak prevention, detection, and control. Opportunities for therapeutic intervention were limited to only a few applications such as herpesvirus infections, influenza, and HIV/AIDS; hence, once a bacterial or fungal infection was excluded, clinicians were limited to providing supportive care for what was presumed to be a viral syndrome. Public health organizations tracked the incidence of viral infections and the development of resistance to the few antiviral drugs in use and provided input to governments and the pharmaceutical industry regarding selection of vaccine targets. More recently, interest in viral diagnostics has burgeoned with the advent of new tools for detection and discovery, global recognition of pandemic risk, high-throughput drug screening, rational drug design, and immunotherapeutics. An additional impetus has been the implication of viruses in chronic illnesses not previously attributed to infection. The objective of this chapter is to review the factors responsible for the rise in awareness of viral infections, methods for diagnosis and monitoring viral infections, and future prospects for improvements in discovery, detection, and response to the challenges of clinical virology.

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1 Emerging Infectious Diseases and Biodefense

In an era when travel and trade are increasingly global, patients with what were once considered exotic infectious diseases restricted to the developing world, like dengue fever, Ebola, or chikungunya, now present in clinics and emergency rooms in North America and Europe. Nonstop flights of less than 24 h connect the world's major airports; hence, physicians must be prepared to expect the unexpected. In New York City, for example, more than 12 million passengers annually pass through John F. Kennedy (JFK) airport from more than 100 international destinations. With this traffic volume in one metropolitan airport alone, it is not surprising that human and stowaway passengers like mosquitoes have been implicated in the transmission of West Nile virus, HIV, influenza virus, *Mycobacterium tuberculosis*, SARS coronavirus, and chikungunya virus. Exotic agents can also transit internationally in legal and illegal (bushmeat) food products and companion animals. The annual traffic in bushmeat through Charles de Gaulle airport in Paris is estimated at 273 tonnes [1]. In work with nonhuman primate, rodent, and bat bushmeat seized at JFK by the Wildlife Conservation Society, EcoHealth Alliance, and the Centers for Disease Control and Prevention, we have found evidence of infection with retroviruses, herpesviruses, and pathogenic bacteria [2]. Illegal importation of companion animals such as birds, primates, and rodents has been linked to outbreaks of poxviruses and Salmonella [3, 4].

Approximately 70 % of emerging infectious diseases are zoonoses—infections that are transmitted to humans from wildlife or domestic animals [5, 6]. The majority of zoonotic diseases can be attributed to anthropogenic change. Loss of wildlife habitat to development and consumption of bushmeat necessitated by poverty or due to cultural preference increases opportunities for cross-species jumps. Global warming may also increase the geographic range of phlebotomus insects like mosquitoes and ticks that serve as reservoirs and vectors for infectious agents [7]. Given that there

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are more than 50,000 vertebrate species, if we assume an average of 20 endemic viruses per vertebrate species, the potential reservoir of vertebrate viruses can be estimated at one million. Although it is unlikely that all of them can be transmitted to humans and cause disease, it is sobering to consider the challenge of detecting and responding even to 1 % of them (10,000 novel viruses).

In the aftermath of the fall of the Twin Towers on September 11, 2001, in New York City, and the anthrax attacks that followed, many western governments became concerned about bioterrorism. Early investments in surveillance for biological weapons gave way to surveillance for emerging infections when sober reflection led to recognition that the latter were more likely threats to public health. However, advances in synthetic biology over the past decade have been so dramatic that clinicians and public health practitioners must again consider the possibility that high-threat known and novel pathogens may arise through deliberate genomic manipulation either in the form of bioweaponeering, inadvertent release of high-threat human pathogens, or legitimate gain-of-function research, whereby low-risk agents become high risk. The scientific and larger communities are currently grappling with the implications of gain-of-function research in the context of experiments designed to understand virulence and transmission of H5N1 (avian) influenza viruses [8–13].

2 Impact of Mechanisms of Pathogenesis on Viral Diagnostics

Establishing a causative link between a virus and disease can be straightforward or complex. In some instances, the virus responsible for the induction of disease is present at a site of organ pathology, and there is precedent for the same or a related virus causing similar disease. A classic example is herpes encephalitis where the detection of herpesvirus sequences by polymerase chain reaction (PCR) in the cerebrospinal fluid (CSF) of a patient with encephalitis provides a clear diagnosis and suggests a specific therapeutic intervention [14]. PCR alone can be inadequate. In West Nile encephalitis, PCR of CSF is less reliable than assays of CSF for IgM antibodies to the virus [15, 16]. In some instances, the footprints of an agent cannot be found in or adjacent to the affected organ but can be detected in other compartments. PCR detection of enterovirus, for example, in the feces of a patient with aseptic meningitis, provides strong evidence of enteroviral meningitis [17]. Despite these examples of success, an etiological agent is not identified by any test in up to 70 % of what is presumed to be viral encephalitis. Similar figures pertain in viral pneumonias.

There are several explanations for surveillance and diagnostic failure. In some instances, the problem is simply lack of access to the appropriate sample. Infectious agents that do not shed into saliva, nasopharyngeal secretions, blood, urine, CSF, or feces may be detected in tissue biopsies. Alternatively, pathogenetic mechanisms may be indirect, or consequences of infection may be delayed obscuring the relationship between the causative agent and the disease.

The most straightforward mechanisms for viral pathogenesis are cellular damage due to replication and lysis, apoptosis, autophagy, or immune responses to proteins expressed on infected cells. However, viruses can also induce systemic damage through cytokine storm resulting in shock, acute respiratory distress, and/or organ failure, cause immunosuppression resulting in opportunistic infection, or break tolerance for self, resulting in autoimmune disease. Infection can be cryptic, impairing differentiated cell functions like hormone secretion, inducing neoplasia, or impairing developmental programs that may not become apparent for months or years. In summary, the challenge in viral diagnostics is to develop strategies for not only detecting footprints of the agent itself in target tissues but also enduring shadows of infection in accessible compartments.

3 Culture

Once the mainstay of viral diagnostics, culture now receives less emphasis in clinical microbiology, chiefly because assays require days rather than hours; thus, information obtained is unlikely to directly impact patient management. Culture nonetheless continues to play an important role in public health as well as basic and clinical research because it enables insights into pathogenesis and the efficacy of drugs, antibodies, and vaccines. The presence of virus can be detected by changes in cell morphology at the level of light microscopy including lysis, rounding, and syncytia formation—fusion of cells as revealed by an increase in size and the presence of more than one nucleus—visualization of pathognomonic structures by electron microscopy; or viral proteins that bind antibodies as revealed through immunohistochemistry or immunofluorescent microscopy. A wide range of cell lines has been established for culturing viruses. Some are immortalized; others are primary cultures that can only be propagated for a few generations. Although some viruses can grow in many cell types, others have fastidious requirements. Some viruses have never been cultured despite implication in disease. In some instances, propagation failure may be overcome by adaptation with serial passaging in the presence of a second, permissive type of cell (cocultivation), the use of antibodies or RNAi to suppress innate immune responses, or cells obtained from genetically modified animals. However, serial passaging can lead to adaptation, including changes in virulence (the capacity of the virus to cause disease) or tropism (the cells and organs the virus

can infect). Indeed, serial passage may be utilized to develop less virulent strains that can be used as vaccines. A potential confound in characterizing samples that may contain more than one virus is that the culture environment can select for the agent that is more fit to replicate—that may or may not be the agent of interest. In an attempt to address this potential confound as well as to propagate viruses that fail to grow in simple cultures, investigators have developed cultures that include more than one cell type. In some instances these complex cultures are designed to replicate the architecture of an organ like the respiratory tract. An alternative to culture in cells is animal inoculation. An advantage of animal inoculation is that the presence of a wide range of cell types is associated with expression of a wide range of receptors that may allow virus entry. Most investigators use suckling mice because their innate immune responses are immature. Others use mice genetically modified to abrogate immune responses.

4 Molecular Assays

Nucleic acid tests (NATs) have largely replaced culture in viral diagnostics due to advantages in cost, speed, and ease of use. Common NAT platforms include polymerase chain reaction (PCR), in situ hybridization, microarray, and high-throughput sequencing.

4.1 Singleplex Assays

These assays, designed to detect individual viruses, are the most common NATs employed in clinical microbiology. They take several forms, but quantitative real-time PCR, wherein nucleic acid replication results in either cleavage or release of a fluorescence-labeled probe oligonucleotide that binds to a sequence region between the regular forward and reverse primers, is the most popular. The continuous ("real time") reading of the reporter fluorescence signal affords these systems with unprecedented dynamic range and low false-negative rate. The required equipment, thermal cycler with fluorescence detector and (laptop) computer for data analysis, is cost competitive, and rugged battery-powered instruments are available for field use. Loop-mediated isothermal amplification (LAMP) tests do not require programmable thermal cyclers [18–20]. In the laboratory, LAMP products are detected in conventional dye-stained agarose gels, but in field applications the estimation of product accumulation through turbidity or dye reading by the naked eye is also possible [21]. The sensitivity of all such assays is highest when primers and/or probe sequences perfectly match the selected single genetic target. Fluorescence-based TaqMan or molecular beacon assays, for example, typically have detection limits of <10 molecules per assay. Although ideal for detecting and quantitating a specific known agent [22, 23], these assays may nonetheless fail with templates of variable sequence composition, especially if this affects the region of reporter molecule binding. This can be particularly challenging in the diagnosis of RNA virus infections as RNA viruses are characterized by high mutation rates and include species with high genetic strain variability. In comparison, consensus PCR assays are less likely to be confounded by sequence divergence but are also less sensitive than the 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 both accommodate sequence variation and be more sensitive than fluorescent or beacon-based singleplex assays. However, whereas in quantitative fluorescence- or beacon-based real-time assays reporter readings are taken indirectly without opening the reaction vessels ("closed system"), nested PCR systems bear a high risk of contamination because of the transfer of (amplified) material from the first to the second, nested reaction [24, 25], even if scrupulous experimental hygiene is observed. Recently, automated (closed) systems have been developed that allow contamination-free transfer between separate reaction compartments of single-use cartridges that may present new opportunities for nested assay design.

4.2 Multiplex Assays

As signs and symptoms of disease are rarely pathognomonic of a single agent, particularly early in the course of an illness, many microbial candidates must be entertained simultaneously. Multiplex NATs provide such an opportunity. The number of candidates considered may range from 10 to 50 with multiplex PCR systems to thousands with microarray platforms to the entire tree of life with unbiased high-throughput sequencing approaches. However, genetic targets compete for assay components in multiplex assays, and thus they may be less sensitive than a singleplex assay. In compensation, multiplex assays provide the advantage of consistently interrogating each sample for a wide range of agents without the selection bias introduced by singleplex testing. This comprehensive coverage is particularly important for surveillance and applications.

4.2.1 Multiplex PCR

Multiplex PCR assays are more difficult to establish than singleplex assays because primer sets may differ in optimal reaction conditions (e.g., annealing temperature or magnesium concentration). Furthermore, complex primer mixtures are more likely to result in primer-primer interactions that reduce assay sensitivity and/or specificity. To advance multiplex

primer design, we developed Greene SCPrimer, a software program that automates consensus primer design over a multiple sequence alignment with customizable primer length, melting temperature, and degree of degeneracy [26].

Gel-based multiplex PCR assays are limited by size differentiation of the amplification products in agarose gels and the concomitant requirement for short product sizes (approx. 90–250 base pairs) to ensure high sensitivity and fidelity [24, 25]. Multiplexing can be achieved in fluorescence- or beacon-based real-time assays to the degree by which different fluorescent reporter emission peaks can be unequivocally separated. At present up to five fluorescent reporter dyes are detected simultaneously, although multiplexing may be increased to some extent by double-labeling strategies and/or melting curve analyses. "Sloppy Molecular Beacons" address this limitation in part by binding to related targets at different melting temperatures [27]; however, they are not suited to detect targets that differ by more than a few nucleotides.

The Bio-Plex (or Luminex) platform employs flow cytometry to detect multiple PCR amplification products bound to matching oligonucleotides that are attached to differently colored fluorescent beads [28, 29]. 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 permit detection of up to approx. 20 genetic targets simultaneously [30–32]; the most commonly used respiratory panels range from 9 to 20 plex [33–37]. Like real-time PCR, these assays rely for assay specificity on a three-oligonucleotide interaction with the target sequence. They are thereby limited in their tolerance for mutated or variant templates when compared to mass spectroscopy (MS)-coupled platforms that require only two oligonucleotide-binding sites, such as MassTag PCR or the Ibis T5000 system.

Two platforms are established that combine PCR with MS for sensitive, simultaneous detection of large numbers of targets. The Ibis T5000 system uses matrix-assisted laser desorption/ionization (MALDI) MS to directly determine the molecular weights of the generated PCR products and to compare them for identification with a database of known or predicted product weights [38–40]. MassTag PCR uses atmospheric pressure chemical ionization (APCI) MS to detect molecular weight reporter tags attached via a photo-cleavable linkage to PCR primers [41]. Whereas the Ibis system or the subsequent electrospray ionization (ESI)-based Plex-ID system requires analytical MS to determine the exact weight of the PCR products and thus depends on advanced mass spectroscopic data analysis, MassTag PCR can be performed using smaller instruments and does not require sophisticated analyses because it only records the known masses of the 40–80 reporter tags used in a given multiplex test. The Ibis system may be able to alert of variants of known organisms via a divergent PCR product weight, but like MassTag PCR, it too requires subsequent sequencing of the product for detailed characterization. A wide variety of syndrome-specific MassTag PCR panels have been developed and applied to the detection of viruses, bacteria, fungi, and parasites associated with acute respiratory diseases, diarrheas, tick-borne diseases, encephalitides/meningitides, and hemorrhagic fevers [41–50].

Although multiplex PCR methods are designed to detect known agents, they can nonetheless facilitate pathogen discovery. MassTag PCR requires only two differently tagged primers per target that may include degenerate positions to address genetic variation of larger taxonomic groups such as a whole species or genus, and its use to investigate influenza-like illness in New York State revealed the presence of a novel rhinovirus clade by the employed conserved enterovirus/rhinovirus primer set [42]. This discovery enabled follow-up studies across the globe wherein this third species of rhinovirus, rhinovirus C, was implicated not only in influenza-like illnesses but also in asthma, pediatric pneumonia, and otitis media [44, 51–63].

4.2.2 Microarray Assays

Whereas multiplex PCR systems support rapid high-throughput diagnosis with highest sensitivity for a limited number of agents, microarray-based systems provide detection of all known pathogens for which sequence information is available, but at the expense of some degree of sensitivity. Modern printing technologies can generate high-quality arrays with several million features, a printing density that enables not only detection of a wide range of infectious agents but also discrimination of medically important types or subtypes. Examples of the latter application include respiratory virus resequencing arrays that identify the different influenza virus HA and NA subtypes [64–68].

The discovery array platforms currently in use are the GreeneChip and the Virochip [69, 70]. The panmicrobial version of the GreeneChip, addressing viruses and in addition pathogenic bacteria, fungi, and parasites, led to the recognition of *Plasmodium falciparum* infection in a case of unexplained fatal hemorrhagic fever during the 2004–2005 Marburg virus outbreak in Angola [70]. A variant of the GreeneChip facilitated recently the implication of Reston Ebola virus in a respiratory disease outbreak on pig farms in the Philippines [71]. In 2003, the Virochip supported the characterization of the SARS coronavirus and was also used subsequently to diagnose parainfluenza virus 4 and infection with a human metapneumovirus variant in cases of acute respiratory disease [69, 72, 73].

Both platforms rely on random PCR strategies to amplify and label nucleic acids for detection. In comparison to multiplex consensus PCR methods employed with some targeted array applications or resequencing arrays, this limits

sensitivity especially with complex sample types. In tissue specimens, for example, the sensitivity may not exceed 10^6–10^7 copies per assay because host and pathogen nucleic acids compete for PCR reagents. Thus, these platforms have been more successful with samples containing comparatively low levels of competing nucleic acid, such as virus culture supernatant, serum, respiratory specimens, spinal fluid, or urine. Improvements in sensitivity to a range of 10^3–10^4 copies per assay have been achieved with methods for host DNA digestion and/or the depletion of host ribosomal RNA (rRNA) prior to amplification through subtraction or use of random primers selected for lack of complementarity to rRNA [74].

In current array platforms, virus detection is achieved via fluorescent reporter systems—either through direct incorporation of fluorescent nucleotides into the PCR product that is bound to the array or with a "sandwich approach" whereby fluorescent-branched chains of DNA are added to the product after it is bound to the array [75, 76]. However, new arrays are in development that will detect viral sequences through changes in electrical conductance. Such platforms would enhance portability by eliminating the need for fluorescent scanners. They may also increase sensitivity and reduce costs by eliminating the need for PCR amplification.

5 High-Throughput Sequencing

High-throughput sequencing has transformed microbiology by enabling discovery as well as diagnostics. Unlike PCR or array methods where investigators must choose the pathogens to be considered or are limited by known sequence information, high-throughput sequencing has the potential to simultaneously detect not only all viruses but also bacteria, fungi, and parasites. Although the technology is presently limited to specialized laboratories, sequencing is becoming increasingly accessible as instruments become smaller, methods become more user friendly, and costs decrease. Over the past 10 years, the cost has decreased 10,000-fold from $5,000 per 1,000 nucleotides in 2001 to $0.5 per 1,000 nucleotides in 2012 [77]. Even more impressive perhaps is the time required to generate sequence data. Projects that required weeks only a decade ago are now completed in hours [78].

Current sequencing platforms 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 redundance before assembly into contiguous sequence streams. These streams, known as contigs, as well as reads that cannot be assembled, are aligned to databases using bioinformatic algorithms that examine homology at the nucleotide and deduced amino acid levels in all six potential reading frames [79]. 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.

6 Proof of Causation

Finding the nucleic acid footprint of a virus is frequently only the first stage in implicating it in disease. There is no functional equivalent in viruses to the pathogenicity islands found in bacteria, wherein specific sequences acquired through horizontal gene transfer confer specific pathogenic properties. The best established criteria for proof of causation in infectious disease were developed in the late 1800s by Koch and Loeffler [80]. Known as Koch's postulates they stipulate that an agent be present in every case of the disease, be specific for the disease, and be sufficient to reproduce the disease after culture and inoculation into a naive host. In the 1930s, Rivers suggested that the development of specific immunity to an agent following the appearance of disease could be used in demonstrating causation [81]. Adapting the original postulates to the molecular era, Fredricks and Relman later established that microbial sequences may be used as surrogates for culturing the actual organism [82]. Lipkin and colleagues recently established levels of confidence in the strength of association between an agent and a disease that considers viral burden and distribution, specific immunity, and prevention or amelioration of disease with use of specific drugs or vaccines [12]. Given the sensitivity of molecular methods, it is imperative that physicians and researchers consider the biological plausibility of an assay result and, where feasible, pursue confirmation with an independent assay, particularly when engaged in pathogen discovery.

7 Future Perspectives

NATs are rapidly replacing classical culture methods in clinical microbiology laboratories. Although some NATs, such as microarrays and high-throughput sequencing, still require substantial investment in equipment and personnel, diagnostic platforms are becoming more accessible and less expensive through miniaturization and improvements in methods for bioinformatic analysis. Systems using handheld microarrays, for example, are in development that will ultimately enable diagnosis at the bedside or in the field. Benchtop sequencers are also in production. It is inevitable that as sequencing costs continue to decrease, clinicians will seek information concerning not only the presence of a single candidate organism but also the predisposition of the host to disease based on genetic factors and coinfections with other microflora. These improvements will bring dramatic benefits to medicine and public health.

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