EXPERT REVIEWS

Expecting the unexpected: nucleic acid-based diagnosis and discovery of emerging viruses

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Extrapolation from recent disease history suggests that changes in the global environment, including virus, vector and human behavior, will continue to influence the spectrum of viruses to which humans are exposed. In this article, these environmental changes will be enumerated, and their potential impact on target-focused, nucleic acid-based diagnostic tests will be considered, followed by a presentation of some emerging technological responses.

Knowledge of the etiology of infectious diseases is crucial for effective treatment. However, the appearance of novel, drug-resistant or previously recognized viruses in unexpected situations poses a challenge to existing diagnostic approaches. Nucleic acid-based assays have demonstrated their worth as highly sensitive and specific tests for known targets, but they require continuous adaptation. This particularly applies to tests for RNA viruses, which, owing to high error rates in RNA polymerases, are genetically variable. The need for adaptation also applies to real-time PCR tests dependent on probe complementarity to virus sequences. If the target sequences are prone to mutation, even closely related viruses can, occasionally, evade detection.

In a changing disease environment, rapidly adaptable assays, capable of detecting novel, unexpected or mutated viruses and quickly providing information on virus drug resistance profile, pathogenicity and host preference, will be necessary. Fully integrated platforms, combining nucleic acid extraction, capture, amplification and analysis, are on the development horizon. The diagnostic spectrum and economy of these platforms will be enhanced by astute assay design (utilizing the latest bioinformatics tools), and by the inclusion of new technologies such as highly multiplexed microsphere, nanowires and real-time assays. These assays will accrue clinical value as specific therapies are developed for viral diseases. New viruses continue to be discovered via metagenomic techniques, including random PCR, new-generation sequencing and arrays. The challenge will be to develop the knowledge and technical means to keep ahead, or at least abreast, of emerging pathogens with our diagnostic tests.

The hypothesis-based diagnostic paradigm

Hypothesis-based diagnosis has been practised for at least three millennia. Leviticus (circa 1440 BCE) provides detailed instructions on the presumptive diagnosis of leprosy [1]. The diagnostic process is explained, based on the shape and color of lesions, the duration of quarantine is specified, re-examination after quarantine is recommended and treatment is described, based on the presumptive diagnosis. Presumptive diagnosis remains crucial for triage. Early, correct deduction can be critical for patient outcome. The WHO estimates up to 100 million dengue infections occur annually with 25,000 fatalities [201]. While there is no specific treatment for dengue, appropriate medical care can save the lives of dengue hemorrhagic fever patients. The ability to rapidly identify the etiological agent and the clinical, immunological and genetic factors that predict disease severity outcome would impact

significantly on the capacity of the overburdened health facilities to manage the disease. The same argument applies for Australian Bat Lyssavirus, Hendra and Nipah viruses, where purified immunoglobulin [2,202] or novel engineered antibody therapeutics are now available [3,4]. In these cases, astute presumptive diagnosis will be critical to facilitate the use of the correct therapeutic antibody or the appropriate public health intervention. Early presumptive diagnosis informs choices between antibacterial, antiviral or antiprotozoal medication; however, this differentiation is difficult, based on circumstances, signs and symptoms (e.g., differentiating between cyclic fever caused by *Streptococcus pyogenes*, several possible arboviruses or malaria).

The hypothesis-based 'rational' approach to diagnostic testing can be summarized as follows:

- Step 1: Suspicion based on signs, symptoms or circumstances

- Step 2: Provisional treatment

- Step 3: Choose or design a target-focused test

- Step 4: Confirm suspicion. If not, return to step 3. If yes, go to step 5

- Step 5: Treat individual

- Step 6: Community-wide intervention

Problems with the hypothesis-based approach

Until recently, most molecular diagnostic procedures are selected as the result of presumptive diagnosis. The nucleic acid-based tests developed over the last two decades have been remarkably successful in accelerating our ability to identify etiological agents. However, the conventional approach to assay design has limitations in the context of a changing disease environment. Most nucleic acid-based tests have been designed to detect single organisms or a small panel of organisms chosen on the basis of presumptive diagnosis. Standard target-focused PCRs or ligase chain reactions, even moderately multiplex tests, will fail when the etiological agent is not part of the panel of tests. Usually, uncommon viral infections are common in immunocompromised patients and novel sequence variants appear. The variations may be in regions of the virus targeted by probes or primers. Novel or known viruses emerge in unexpected places or under unforeseen circumstances. Moreover, in the absence of sequence information, the design of primers and/or probes for new tests to detect the new disease is time consuming. By the time a new probe has been added to the suite of target-focused tests, the disease outbreak may have run its course. Indeed, many deaths from viral encephalitis in the UK are not attributed to a specific etiologic agent [5] so there is evidently room for improvement, which presents an opportunity for broad-spectrum molecular diagnostics, not narrowly focused on the basis of presumptive diagnosis.

Factors in the environment that have the potential to influence the spectrum of viruses to which *Homo sapiens* is exposed will be summarized in the following section. The potential impact of each factor on target-focused, nucleic acid-based diagnostic tests will be discussed, before the presentation of examples of emerging technological responses.

The reservoir of unknown viruses

Viruses constitute a vast reservoir of genetic diversity [6]. Surveillance programs continue to discover new viruses. The National Arbovirus Monitoring Program in Northern Australia samples hundreds of 'sentinel' cattle herds and field-trapped insects. Thousands of viruses have been collected, cultured and stored, but not identified, and the pathogenic potential of the vast majority remains unknown. An example of recent work in this area is the application of PCR-select cDNA subtractive hybridization, to characterize several new *Rhabdoviridae* from *Culicoides* spp. (biting midges). Antibodies to one of these viruses were found in cattle and macropods [7]. Among the new viruses discovered in the last 15 years are highly pathogenic avian influenza (H5N1) and Hendra/Nipah. Severe acute respiratory syndrome-associated coronavirus, human bocavirus, novel human parvoviruses, human polyomaviruses, human metapneumoviruses, zoonotic human T-cell lymphotropic viruses (HTLVs), herpesviruses and flaviviruses, which will be mentioned later in this article.

Climate change

It is reasonable to suggest that climate change will impact on the distribution of disease vectors and viruses [8]. However, the geographic directions of change and local impact due to climate are unlikely to be predictable in a simple, latitude-dependent manner. Change in vector distribution, for example, the incursions of *Aedes albopictus* from Torres Strait into northern Australia, are likely to lead to permanent colonization [9]. These mosquitoes have the capacity to spread a suite of viruses (West Nile virus [WNV], yellow fever virus, St. Louis encephalitis, dengue fever and chikungunya virus [CHIKV]) into higher latitudes. Irrespective of whether this change is related to climate change, to the rapid adaptation of mosquitoes to cooler climates or the adaptation of viruses to new vectors [10,11], the need for continued adaptation of our diagnostic methods is evident. Extreme climatic events, such as flooding, have an impact on the local disease incidence, and moreover, flooding provides water corridors for the distribution of insect vectors over large distances.

Changes in the artificial environment

Anthropogenic change of the physical environment has an impact on arbovirus distribution and the frequency of arbovirus-related illnesses. For example, change in water tank usage has been proposed to have had a major effect on the distribution of dengue fever in Australia since the early 20th Century. Russell and colleagues pointed out that, although the current range of *Aedes aegypti* is restricted to Queensland, the historical distribution was wider and both the vector and virus were formerly distributed all across Australia [12]. A return to previous distribution of dengue could occur as a result of factors other than climate change (e.g., changes in water management or vector-control programs). Irrigation and deforestation have had a major impact

on the occurrence of epidemics of Rift Valley fever in Africa. Urbanization in Asia has had an impact on dengue outbreaks. Gould and colleagues have provided a comprehensive review of the range of zoonotic arboviruses in Western Europe [13,14]. There is strong serological evidence that WNV, Usutu and sindbis virus already circulate in the UK. The authors concluded that climate change is probably the most important requirement for emergence of arthropod-borne diseases such as dengue fever, yellow fever, Rift Valley fever, Japanese encephalitis and blue tongue in the UK. However, the unavailability of reservoirs will also be a limiting factor (e.g., the natural reservoir for yellow fever virus [YFV] is tree-dwelling monkeys and their associated mosquitoes). One unexpected consequence of global attempts to control *Ae. aegypti* appears to have been the progressive displacement of *Ae. aegypti* by *Aedes albopictus* (the Asian tiger mosquito). The global spread of *Ae. albopictus* has also been facilitated by tyre shipments. *Ae. albopictus* is now established in most tropical, subtropical and temperate regions [15,16]. As *Ae. albopictus* continues to spread, the distribution of dengue, chikungunya and other viruses will change. New diagnostic tests need to anticipate this development.

Adaptation of viruses to new vectors

Mosquito-borne flaviviruses have a propensity to adapt to new mosquito hosts, so they possess the potential to emerge in areas inhabited by the new host [17]. WNV has been isolated from more than 60 mosquito species in North America, and WNV, YFV and St Louis encephalitis virus (SLEV) have also been isolated from ticks, which is indicative of vector promiscuity [18]. CHIKV appears to have acquired an *A226V* mutation in the E1 envelope, which improves its transmission competence for *Ae. albopictus*. This adaptation is postulated to have facilitated the 2007 epidemic in northern Italy, and may have facilitated the outbreak in La Reunion. *Ae. aegypti* has long been considered to be the natural and most effective vector of CHIKV for humans, but this mosquito species has not yet become established as a significant vector of CHIKV, either in Italy or La Reunion [8]. *Ae. albopictus* has gradually been dispersing worldwide and is present and active in Italy and La Reunion, but was not considered to be an effective vector of CHIKV until the virus mutated in the E1 protein (A226V). This improved the vector competence of *Ae. albopictus* for CHIKV. The *A226V* mutation appears to have arisen on several independent occasions in CHIKV from different geographic regions, implying convergent evolution, presumably under the selective pressure of the tiger mosquito. De Lamballerie *et al.* describe three distinct examples of convergent evolution that were identified during an approximate period between 2005 and 2007 [19]. These were identified in the regions of La Reunion/Mauritius/Madagascar, India (this covers the Italian virus which was introduced from India), and Cameroon and Gabon. Populations of *Ae. albopictus* in Florida (USA) are able to transmit CHIKV [20], thus diagnostic test panels should be expanded to include this pathogen and facilitate its differentiation from other related alphaviruses circulating in the Americas. Moreover, rapid and precise diagnosis would

increase the likelihood of identifying the associated vector species. It would be advantageous if these new diagnostic tests could incorporate detection of the *A226V* mutation as a predictor of the relative ease of transmission in regions outside the range of *Ae. aegypti*.

Rapid movement by humans

The arrival of a CHIKV-infected traveller from India in Italy in 2007 triggered an outbreak of more than 200 cases of chikungunya fever [21] . All dengue fever outbreaks in Australia to date have been seeded by infected travellers. For example, dengue virus serotype 2 circulating in Queensland during the 2008–2009 outbreak was imported by an Australian traveller returning from Indonesia [22].

In an elegant study of the emergence and spread of drug resistance, Meijer and colleagues monitored the West-to-East movement across Europe of H1N1 influenza A virus, during the 2007–2008 season [23]. The increase in frequency and distribution of oseltamivir resistance, due to the *H275Y* mutation in the neuraminidase gene segment, was monitored over the same period. The fitness of the oseltamivir-resistant virus was attributed to the occurrence of the *H275Y* mutation in a new genetic background provided by the A/Brisbane/59/2007 drift variant. The oseltamivir-resistant virus was estimated to have dispersed across Europe at 1° longitude per 0.156 weeks [23] which, at latitude 45°, corresponds to 73 km per day, a rate consistent with human travel.

Movement of animals & vectors: contact between humans & animals

Movement of animal hosts and vectors changes pathogen distribution. Bats host a wide range of zoonotic agents, including lyssa, Hendra and Nipah SARS-like corona, Marburg, Ebola, Japanese encephalitis virus (JEV), Kyasanur Forest disease virus, astroviruses, WNV [24] and Australian Bat Lyssavirus. There has been a contemporaneous emergence of several zoonotic viruses in the last two decades. The transmission of viruses to humans has been facilitated by bidirectional habitat encroachment and increasing frequencies of contact between bats and humans, sometimes involving an intermediate domestic animal host (e.g., pigs in the case of Nipah virus, horses in the case of Hendra virus and civets in the case of SARS). Satellite telemetry has demonstrated that bats travel long distances (e.g., between Australia and Papua New Guinea [25]). Birds play a role in the dispersal of arboviruses such as sindbis virus, SLEV, WNV and Usutu virus (USUV) [13]. USUV, hitherto found only in Africa, emerged in Vienna in 2001 and was associated with the large-scale death of birds [26], initially incorrectly suspected to be an outbreak of WNV encephalitis (see later).

Some rare zoonoses exhibit extreme virulence in humans. Herpes B virus (*Herpesvirus simiae*) is a case in point, where the human survival rate is only 25% and most patients die within a few weeks [27]. Several human herpesviruses, cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex (HSV-1) have been implicated in retinitis or retinopathy, with similar

presentation. Prompt differential diagnosis from the vesicular lesions is crucial in these cases, but herpes B virus is not routinely included in the standard suite of targets in multiplex real-time PCR-based herpes diagnostics [28]. It would be missed by most assays, as they are dependent on target-focused probes.

The primate origins of HIV and HTLV-1 are well established. Recent studies have provided evidence for frequent and regular transmissions of retroviruses from captive and wild animals to exposed humans [29,30]. Three new primate retroviruses, simian foamy virus, HTLV-3 and HTLV-4, have been identified in humans exposed to blood and body fluids of primates (either through laboratory contact, hunting or keeping of wild pets) [31]. Regular transmission from primates to humans suggests that viral adaptation to new hosts plays an important role in cross-species transmission and large-scale emergence in humans. This finding has important implications for the design of diagnostic tests employed in public health surveillance, because of the difficulty in predicting which retrovirus will emerge in the future in humans.

Mutation & recombination events in viruses

Viruses exist as quasispecies [32]. Infected humans and animals host a diverse population of viral nucleic acid sequences; the raw material for rapid evolution. RNA viruses, including retroviruses, have much higher mutation rates than DNA viruses [33,34], increasing the complexity of generating effective probes for nucleic acid amplification tests. This can result in assay 'drop out'. This has been a problem with influenza A diagnostics targeting hemagglutinin (HA) and neuraminidase (NA) segments. It is exemplified by the necessity for *ad hoc* test development for the 2009 H1N1 human influenza of swine origin, the 'swine influenza virus real-time detection panel', and its authorization for emergency use by the US FDA. This test was subsequently replaced by an improved version developed by the CDC, as more H1N1 specimens became available [203]. The problem of drop out continues to plague commercial and in-house assays [35]. Whiley and colleagues cite several examples of PCR or reverse-transcriptase (RT)-PCR-based virus diagnostic assays that are prone to false-negative results caused by sequence variation. This list includes commercial assays for HIV-1, human parvovirus B19, and in-house methods for rabies and cytomegalovirus [35]. This calls for astute primer design or the use of multiple gene targets within a single organism (e.g., combining PCRs for the *por A* and *ctr A* genes in *Neisseria meningitidis* [36] or for the *HA* and *NA* genes for influenza A detection) [37]. The challenge to the designer of an influenza diagnostic test is the same as the challenge faced by the designer of influenza vaccines, where the vaccine needs to be reformulated annually to account for antigenic drift and also when antigenic shift is detected.

Mutation, reassortment or recombination can confer a selective advantage on the virus. This might result from resistance to antiviral drugs, evasion of host immune response, increased replicative efficiency or survival in new host species. In the recent (2008/2009) Italian outbreak, WNV has acquired a *T249P* mutation in the NS3 protein, associated with avian virulence and human outbreaks. The mutation is predicted to increase the high temperature stability of the WNV helicase [38]. For some viruses (e.g., influenza A virus) the molecular signatures associated with clinically important phenotypes are well characterized and could be incorporated into first-line diagnostic tests, providing critical and timely information on host preference, pathogenicity and drug resistance.

Recombination has been reported between a wide range of homologous RNA viruses, including picornaviruses [39], alphaviruses [40], the GB virus C/hepatitis G virus [41], hepatitis C virus [42] and JEV [43,44]. No evidence for recombination was found for recombination in WNV, YFV, tick-borne encephalitis virus (TBEV) [45] or SLEV [46].

Recombination has been discussed in the literature because of its potential impact on virus emergence, pathogenicity, evolution and vaccine efficacy [17], and its implications for the safety of live vaccines [47]. From a diagnostic point of view, it should be considered as a potential source of drop out in target-focused tests, particularly those using PCR primers or real-time probes that are exact matches to the target sequences.

Recent manifestations of the dynamic disease environment

West Nile virus appeared for the first time in the Americas in August 1999 and within 7 years had caused approximately 10,000 cases of neuroinvasive disease in North America alone, with a 10% fatality rate among clinically apparent encephalitic cases [48]. WNV outbreaks have been recorded in birds, horses and humans in Europe and Russia since the 1960s. The most recent recorded outbreak in Italy was reported in 2008/2009 [38]. For many years, CHIKV was regarded as an African virus that caused occasional localized outbreaks in human communities closely associated with the forest regions where the virus circulates among *Aedes* spp. Prior to 2005, the virus was also spasmodically associated with outbreaks of chikungunya fever in India. In 2005, the virus appeared for the first time in La Réunion and the Comoros Islands and quickly became established as both epidemic and endemic. Subsequently, it dispersed to India and many other parts of Malaysia where epidemics are now common and involve millions of humans annually. For the first time, this virus also recently caused epidemics in Italy. Thus, CHIKV is now endemic/epidemic in the southern Oceans and Asia and appears to be moving into southern Europe [49,50]. USUV was detected for the first time in Vienna in 2001, where it caused fatal encephalitis in wild birds. This virus has now been detected in several southern European countries and in 2009 was identified as the etiological agent for a case of human neuroinvasive disease [51]. Bluetongue virus (BTV) is now endemic in southern Europe and arrived for the first time in northern Europe in 2006. Indeed, BTV crossed the English Channel and caused outbreaks in the UK in 2008 before it was controlled using a combination of quarantine, controlled importation measures and vaccine usage. At the time of writing, BTV has not reappeared in the UK. Crimean-Congo hemorrhagic fever has recently occurred for the first time in southern Europe. Rift Valley fever has, on two occasions, moved out of Africa into the Arabian Peninsula. Since the discovery of Hendra virus in Australia in 1994, and Nipah virus in 1998, there have been 199 cases of hemorrhagic fever due to one or other of

these viruses [204]. In South America, dengue virus has expanded its range to Argentina. In September 2010, two independent cases of autochthonous transmission of dengue fever and two of chikungunya fever occurred in south-eastern France [52]. Clearly, these events have implications for Europe and consequently for innovative and rapid design of nucleic acid-based virus diagnostics.

The attributes of nucleic acid-based diagnostic tests for an environment of emerging diseases

What are the implications of the emerging disease environment for the design of diagnostic tests? Modern-generation diagnostic tests must be readily adaptable to, and capable of, detecting and identifying both known and unknown, even variant or novel, viruses in field samples.

Technological approaches to broad-spectrum virus diagnosis

There are fundamentally three levels or categories of diagnosis, based on the level of confidence in our hypotheses regarding the etiological agent:

• Specific identity of the etiological agent is suspected

• Identity of the etiological agent is suspected at the level of the genus, family or group of agents that cause encephalitis, hemorrhagic fever or respiratory infections, and so on

• Identity of the etiological agent is completely unknown

Under the first scenario, the diagnosis could potentially be carried out via tools that incorporate:

• Targeted multiplex real-time PCR

• A suite of targeted multiplex PCRs

• PCR-microsphere panels

Under the second scenario, the diagnosis could potentially be carried out via tools that incorporate degenerate PCR (e.g., targeted at genus level), followed by:

• Sequencing

• Simple chip/array

• Microsphere detection

• Mass spectrometry

Under the third scenario, or when other focused assays have failed, the diagnosis could potentially be carried out via tools that incorporate:

• Metagenomics

• Random PCR

• Pyrosequencing

• Array-based detection

Examples

Specific identity of the etiological agent is suspected

Multiplex real-time PCR

Real-time PCR has rapidly become the favored method of choice in clinical virology laboratories. It is routinely used in target-focused tests for many viruses, including HIV-1 [53], parvovirus [54], BTV [55], WNV [56], dengue virus [57], influenza A virus, influenza B virus and at least eight human herpes viruses. Multiplexing is utilized in some of these tests, both to enable coamplification of internal and external controls along with the target viral nucleic acid (e.g., [55]), as well as for reasons of economy and work flow, to include several of the detectable targets in one or two reaction tubes. The level of multiplexing can be increased by various strategies. One method (utilized in [28]) entails dividing the extracted nucleic acid samples into more than one tube and using sequential real-time reactions after the positive reaction is identified by melt-curve analysis in one of the tubes. By this means it has proved possible to detect and differentiate HSV1, HSV2, VZV, Epstein–Barr virus (EBV), CMV, human herpes virus (HHV)6, HHV7 and HHV8. As mentioned earlier, some of the rarer, yet highly pathogenic, herpes viruses (e.g., herpes simiae virus) are not included in these panels. As many of these multiplex real-time PCR assays are dependent on multiple, exact-match primers directed to single target genes, they are vulnerable to sequence variation and 'drop-out of signal'. Another strategy to increase the level of multiplexing is to utilize GC-rich extensions on the tails of PCR primers, allowing discrimination of amplified targets by melt-curve analysis. Lo et al. used this approach, combined with the use of two fluorophores and the intercalating dye SYBR® Green [57]. Using this hybrid strategy, the authors were able to detect and differentiate dengue virus serotypes 1, 2, 3 and 4. However, the assay was not designed to detect JEV, which cocirculates with dengue in many locations in Asia.

Multiplex PCR-microsphere respiratory virus panels

The other target-focused approach that is used to identify one of a candidate panel of viruses is microspheres/microbeads. Microspheres enable capture and spatial separation of PCR products, and the products can be 'addressed' via the unique correspondence between the oligonucleotide capture sequence on the microsphere and the color of the microsphere.

There are now three commercially available respiratory virus panels that rely on targeted multiplex PCR (ResPlex II v2.0 [Qiagen], MultiCode®-PLx [EraGen Biosciences] and xTAG® [Luminex]). Balada Llasat et al. very recently evaluated these three systems [58]. The respiratory panel developed by Mahony et al. detects 20 human respiratory viruses or virus subtypes and is now commercially available as the Luminex xTAG system [59]. It relies on multiplex PCR using 14 virus-specific primers, followed by capture of the oligonucleotide 'tagged' PCR products onto universal oligonucleotide tags on the microspheres, followed by target-specific primer extension, during which a phycoerythrin label is incorporated into the extension product. The microspheres pass through a flow cell and the

signals are detected by a red laser to identify the microspheres and a green laser to measure the phycoerythrin localized to the microsphere. The list of detected viruses includes influenza A (subtypes H1, H3 and H5), influenza B, respiratory syncytial virus A and B, metapneumovirus, rhinovirus, parainfluenza 1–4, severe acute respiratory syndrome-associated coronavirus, and four other coronaviruses and adenoviruses of unspecified type. Nolte and colleagues contemporaneously developed a very similar system, utilizing modified nucleotides to label the microsphere-captured primer extension products [60]. The latter system, now commercially available as the MultiCode-PLx is designed to detect 17 respiratory viruses. All three of the commercially available multiplex PCR panels exhibited superior sensitivity compared with virus culture methods [58]. Although all of these systems cast a relatively broad net, they are target-focused, use target-specific primers and are therefore susceptible to drop out, or failure to detect variant viruses.

To illustrate this point, the xTAG respiratory virus panel, in addition to detecting influenza A, identifies the subtype of seasonal influenza A as H1 or H3. However, the subtype of the novel influenza A virus of swine origin (2009 outbreak) could not be identified. This emergence of an untypeable influenza A virus, using the xTAG panel, was cleverly turned from a 'negative' to a 'positive' test by Ginochio and St George, who demonstrated that untypeable influenza A virus was, at least for the 2009 outbreak, statistically very likely to be H1N1 [61]. A more robust diagnostic solution (e.g., redesign of the xTAG panel) is needed, because any future change in subtype prevalence will invalidate that conclusion.

Ginocchio *et al.* also compared the xTAG system with the BinaxNOW® influenza A and B test (Inverness Medical International, Cranfield, UK) and the 3M™ Rapid Detection Flu A + B test (3M, St Paul, MN, USA; both antigen-detection tests) [62]. The xTAG panel demonstrated far superior sensitivity for inferred detection of novel H1N1 influenza of swine origin (i.e., for detection of influenza A virus that was untypeable using the xTAG panel). However, the xTAG respiratory virus panel should be modified to allow unambiguous subtyping of influenza A virus in order to confirm these provisional comparisons with other tests.

These examples serve to demonstrate the types of problems that arise when developing diagnostic tests designed to identify specific viruses.

Identity of etiological agent is suspected at the level of genus, family or group of agents that cause common clinical syndromes

Several flaviviruses cause severe encephalitic, hemorrhagic and/or febrile illness in humans. Typically, symptoms include sudden onset of fever, anorexia and headache. Vomiting, nausea, diarrhea, muscle aches and dizziness may also occur. Clinical symptoms are not distinctive of infection by a particular virus (see TABLE 1). Various arboviruses cocirculate in geographically overlapping regions, with incursions of exotic flaviviruses into unanticipated regions becoming more common (see later), posing a difficult

challenge for diagnostics. The pan-genus diagnostic approach has been utilized for three decades in antibody-based assays, with pan-flavivirus monoclonal antibodies developed in the early 1980s [63]. Follow-up immunological tests were necessary for species identification but these are not always definitive (see later for the example of the failure of West Nile differentiation from USUV by immunological methods). A genus-level nucleic acid-based diagnostic, capable of detecting any member of the genus, would be a useful front-line diagnostic tool, particularly if interfaced with an array facilitating rapid species identification. Such tools are currently being developed.

To address this challenge, a range of broad-spectrum RT-PCRs have been developed (e.g., Gaunt and Gould [64], Moureau *et al.* [65], Maher-Sturgess *et al.* [66] and Fischer *et al.* [67]). Maher-Sturgess *et al.* developed a pan-genus, one-step RT-PCR, with an extended range of positive reactions compared with earlier publications [66]. Sequence alignments of 490 flavivirus sequences were used to select highly conserved sites in all known members of the genus. A computer program capturing the method of Zheng *et al.* [68] was then used to chart the sequence stability in the selected, conserved flavivirus sites over the time period since the first flavivirus was entered into the National Center for Biotechnology Information database. This program (dubbed 'Lu-Tze') identifies the least rapidly changing sequences among the initially selected group of conserved sites. A region in the *NS5* gene was selected and redundant (mixed-base) primers were designed [66], amplifying a product rich in phylogenetic information and suitable for downstream analysis using array technology (see later). This assay was effective in detecting all 66 tested flaviviruses from all three groups: mosquito-borne, tick-borne and no known vector, as well as some recently isolated variant viruses (i.e., a variant Edge Hill Virus, a member of the YFV group) [69].

Bioinformatics innovation

The RT-PCR was interfaced with a prototype array detection system that utilized a novel probe design [65]. These 'dichot' or 'binary' probes successively divide a target population of sequences into two populations [70]: those that react with the probe and those that do not (FIGURE 1). As each probe generates a yes/no (binary) answer, they can be described in terms of a base 2 logarithm, such that $Y = 2^X$, where X is the number of probes and Y is the maximum number of species that can be differentiated. For example, ten probes differentiate $2^{10} = 1024$ species and 20 probes differentiate >1 million. This system generates a binary barcode. The discovery of a new nonzero barcode flags a new but related virus. For example, if one compares the binary barcodes of two viruses on a dichot array consisting of nine spots, 100100100 and 100100101, these different codes can be interpreted as revealing three regions of similarity and one region of nonsimilarity between two viruses. This type of array detection yields an immediately expandable test. Newly discovered viruses will be those generating a new barcode on the existing array. The new barcode can be entered into a pattern-matching database and a new test is rapidly available. This and other array-based, nontarget-focused detection methods are examples of adaptive

Table 1. The overlapping spectrum of possible signs and symptoms for some of the clinically significant flaviviruses.

Virus	Symptom							
	Fever	*Headache*	*Nausea/ vomiting*	*Dizziness/ disorientation*	*Encephalitis*	*Myalgia*	*Rash*	*Hemorrhagic fever*
MVEV	√	√	√	√	√	√	√	
YFV	√	√	√	√	√	√	√ Jaundice	√
DENV	√	√	√	√	√	√	√ Jaundice	√
WNV	√	√	√	√	√	√	√	
JEV	√	√	√	√	√			
KUNV	√	√	√	√	Rare	√		
USUV	√	√		√	√		√	
TBEV	√	√	√	√	√	√		
SLEV	√	√	√	√	√	√		
KFDV	√	√	√	√	√	√		√
OHFV	√	√	√	√	√	√	√	√
AHFV	√	√	√	√	√	√		√

Not all symptoms or signs occur in all cases and there are febrile or meningoencephalitic forms of some of the diseases (e.g., TBEV), which present and progress differently. For several of these viruses, a significant proportion of infections can be asymptomatic (e.g., WNV). Substantial fatality is associated with symptomatic JEV, MVEV, AKHV, KFDV, WNV, DENV and YFV in unvaccinated populations.
AHFV: Alkhurma hemorrhagic fever virus; DENV: Dengue virus; JEV: Japanese encephalitis virus; KFDV: Kyasanur Forest disease virus; KUNV: Kunjin virus; MVEV: Murray Valley encephalitis; OHFV: Omsk hemorrhagic fever virus; SLEV: St Louis encephalitis virus; TBEV: Tick-borne encephalitis virus; USUV: Usutu virus; WNV: West Nile virus; YFV: Yellow fever virus.
Data from [203,204].

diagnostic tests. Wang *et al.* demonstrated that nucleic acid can be recovered from arrays and used for detailed characterization of new viruses [71].

Moureau *et al.* also developed a one-step RT-PCR method for universal (more correctly, pan-genus) detection of flaviviruses [65]. The primers (PF1S/PF2R) were a modification of degenerate primers described earlier [72–74]. Their assay was extended to develop a real-time RT-PCR version of the test, using the SYBR green system. The real-time assay, when positive, was followed by direct sequencing using the amplification primers. This test exhibited exquisite sensitivity and, notably, was capable of detecting cell fusing agent virus in mosquito pools and Ngoye virus from crushed ticks.

The protocols of Maher-Sturgess *et al.* [66] and Moureau *et al.* [65] avoid the use of nested PCR, a feature of many earlier assays, which is prone to contamination.

Previously found only in Africa, USUV appeared in Vienna, Austria, in 2001. The association with extensive bird mortality led to a presumptive diagnosis of WNV [26]. Although serology and histochemistry was positive for WNV, samples were negative in a target-focused West Nile virus PCR [48]. Accordingly, a 'universal' RT-PCR was developed using degenerate primers based on the sequences of several mosquito-borne flaviviruses [26]. The use of the new RT-PCR, targeting the NS5 region, followed by sequencing, led to the identification of USUV. Similarly, in a 2009 case of neuroinvasive infection [51], molecular tests of cerebrospinal fluid were negative for CMV, HSV1/2, EBV, adenoviruses, parvovirus B19

and WNV. The use of a hemi-nested degenerate PCR for genus *Flavivirus* [75] was necessary to amplify the virus and enabled subsequent identification of USUV by direct sequencing [50].

An expansion of arbovirus surveillance and reporting systems was implemented in North America following the appearance of WNV. ArboNET collects and reports data from humans, mosquitoes, birds, mammals and sentinel chickens. These data are integrated into a single reporting system [76]. Similar surveillance expansions have taken place in Europe after WNV and USUV outbreaks [38]. Broad-spectrum molecular tests such as those previously described should make a significant contribution to such programs.

Influenza A virus possesses molecular signatures that determine host preference, pathogenic potential and drug resistance. These signatures are located in the *HA* gene segment, the *PB2* gene segment, the *NA* gene segment and the *M* gene segment. Although the association between signature and phenotype is well established, the signatures have not yet been utilized in front-line diagnostic tests, which currently give little more than rudimentary information about the HA and NA type. In response to this need, Castillo Alvarez *et al.* developed a degenerate primer, single-step, touch-down RT-PCR to detect all NA subtypes, amplifying the region of the *NA* gene segment containing the molecular signature (H275Y) encoding oseltamivir resistance, and the PB2 segment from all influenza subtypes containing molecular signatures for pathogenicity [77,78]. A second weakness of current front-line

diagnostic tests is that most of the commercial and in-house assays target a limited range of HA and NA types. Moreover, they are prone to drop out due to sequence variation in primer and probe regions [35]. In response to this problem, a more robust, albeit target-focused, real-time RT-PCR was developed to detect H1N1 of swine origin. It was improved by using RT-PCR to amplify two gene segments, the *HA* and *NA* genes [37]. This assay detected the first case of novel H1N1 influenza in Australia.

RT-PCR amplification followed by microsphere array detection
Fischer *et al.* have also utilized a universal PCR for flaviviruses [67]. A first phase of symmetric PCR was followed by a second round of asymmetric PCR, followed by capture of PCR products onto Luminex beads preconjugated to primers specific for 12 flaviviruses and four alphaviruses. This detection assay showed better sensivity than TaqMan® PCR for Kunjin and Japanese encephalitis viruses. A disadvantage of this type of microsphere panel (a disadvantage shared with the respiratory virus panels discussed previously) is that they typically contain only one virus-specific probe per virus, rendering them susceptible to drop out in the event of sequence change.

Degenerate RT-PCR amplification followed by mass spectrometry
There are more than 29 species in the genus alphavirus and many subtypes; an intractable challenge for target-focused diagnostics. Eshoo *et al.* developed a multilocus broad-spectrum RT-PCR utilizing degenerate oligonucleotide primers followed by electrospray ionization mass spectrometry (ESI-MS) that identifies alphaviruses by base composition [79]. A small set of mixed-base primer pairs targeted conserved sites in the alphavirus RNA genome. Base compositions from the amplicons could unambiguously assign the species or subtype of 35 of the 36 isolates of Old and New World alphaviruses. The assay was used to detect alphaviruses in naturally occurring mosquito vectors collected from locations in South America and Asia. One mosquito pool from Peru contained an alphavirus with a distinctive mass spectrum signature. Subsequent sequencing confirmed that the virus was a member of a new subtype of the *Mucambo virus* species (subtype IIID in the Venezuelan equine encephalitis virus complex). This high-throughput assay is useful both for surveillance and discovery of uncharacterized or emerging viruses.

Identity of etiological agent is completely unknown: no hypothesis
At this level, the metagenomics approach is adopted whereby randomized amplification is followed by undirected or semidirected sequencing. Petrosino *et al.* defined metagenomics as "culture-independent studies of the collective set of genomes of mixed microbial communities" [80]. It is an approach that can be used for pathogen identification in mixed microbial communities in the absence of an hypothesis, or when the first two diagnostic approaches listed in the section entitled 'Technological approaches to broad spectrum virus diagnosis' have failed. Metagenomic approaches have the power to sample the spectrum of known and novel viruses present in clinical samples. The detection of novel viruses facilitates follow-up research to determine the role of these novel viruses in etiology and epidemiology.

The studies of Van den Hoogen *et al.* [81], Allander *et al.* [82], Gaynor *et al.* [83], Finkelbeiner *et al.* [84], Nanda *et al.* [85], Uhlenhaut *et al.* [86,87] and Epstein *et al.* [24] all involve the use of various versions of random amplification PCR or degenerate oligonucleotide PCR, prior to cloning into plasmid vectors and sequencing [84] or direct high-throughput pyrosequencing [24,87].

The work of Gaynor *et al.* is instructive on the general principles involved in the metagenomic approach and how it fits into the diagnostic armory [83]. A nasopharyngeal aspirate (NPA) was obtained from a child with pneumonia. A total of 17 target-focused PCR assays for known respiratory viruses produced negative results. Total nucleic acid from the NPA was randomly amplified using the sequential, random primed 'AB' protocol of Wang *et al.* [71]. The primer B products were cloned and 384 clones were sequenced using standard dye terminator chemistry. The strategy resulted in 327 human sequences, six known bacterial sequences and six viral sequences, all with limited homology (34–50% predicted amino acid identity) to known polyoma viruses. This led to the discovery of a novel polyoma virus. Specific primers were then designed and prevalence studies undertaken on 1254 respiratory samples, revealing a 3% prevalence. Finkbeiner *et al.* used the same approach, including the random primed 'AB' amplification method, followed by cloning and sequencing to identify a spectrum of known and novel viruses from human diarrhea, including highly divergent astrovirus and, unexpectedly, sequences from nodaviruses not hitherto associated with infection in mammals [84].

Epstein *et al.* utilized a random octamer amplification combined with an unbiased pyrosequencing approach as a discovery tool, to screen sera from *Pteropus* bats for the presence of known or novel infectious agents [24]. PCR products greater than 70 bp in size were selected by column chromatography and then ligated to linkers for unbiased pyrosequencing. By this means, a novel GB-like flavivirus was discovered.

Metagenomics is not yet widely used in clinical virology laboratories, but work in bacterial pathogen identification [88] has mapped a path for the introduction of metagenomics into routine pathogen testing in a clinical setting. One of the challenges highlighted by Luna *et al.* is setting the decision criteria for sending samples for pyrosequencing, rather than utilizing more routine, target-focused assays [88]. Even in those cases where metagenomics and pyrosequencing is not part of the routine work flow and is rarely used (i.e., only when all routine assays are negative), the results of the metagenomic approach could be rapidly introduced into routine clinical diagnostics, if only as an expanded version in the panel of target-focused tests.

Metagenomics approaches: random amplification followed by microarray detection
The road to incorporating metagenomics into the clinical virology lab may be smoothed by combining random amplification strategies with densely tiled arrays or dichot arrays (see earlier).

Wang *et al.* combined the random primed AB PCR protocol with a densely tiled array containing the most highly conserved 70-mer sequences from every fully sequenced genome in GenBank [71]. By this means, rapid identification by pattern-matching is possible, and novel viruses can be discovered by simply scraping hybridized viral sequences from the array. A novel coronavirus was discovered by this method [71].

Although pathogen arrays have proven their utility for discovering novel viruses, there have been technical problems related to accuracy and sensitivity, resulting in resistance to their use in routine patient care. Wong *et al.* demonstrated that amplification efficiency by random primers is the crucial determinant of the probe hybridization signal [89]. They developed an algorithm to predict the random primed viral amplification efficiency score [90]. In order to correlate the predicted virus amplification efficiency score with the array hybridization signal, they utilized Nimblegen array synthesis technology to detect 35 RNA viruses, using 40-mer probes tiled across the full length of each virus genome (390,482 probes, including replicates of each probe and controls). The prediction algorithm was used to design an amplification efficiency score optimized primer that had the highest score for all 35 viruses represented on the array. By utilizing the optimized amplification, the microarray platform identified pathogens with 76% sensitivity and 100% specificity relative to real-time PCR.

CombiMatrix corporation have developed sequencing microarrays, including a broad-scan microarray for subtyping influenza A virus [91]. The system is technologically elegant; however, the multiplex RT-PCR amplification of influenza virus nucleic acid prior to hybridization depends on the use of target-specific reverse primers [91] and, moreover, the probes on the array are unique to each of the target subtypes. This configuration means that the system is potentially vulnerable to drop out and will need to be redesigned when new subtypes arise or a mutation arises in regions of the virus recognized by the primers or probes. Bolotin *et al.* evaluated the CombiMatrix influenza detection system, comparing it with the FDA-approved Luminex respiratory virus panel (see earlier), for subtyping influenza A virus [92]. Although the limit of detection for the CombiMatrix test was three orders of magnitude greater than the respiratory syncytial virus panel, the sensitivity for detecting either H1 or H3 seasonal influenza viruses was still 95% relative to the Luminex respiratory virus panel.

Nanotechnology & biosensors

The core elements of biosensors are the amplification and detection technologies (that may be built using nanotechnology). However, assay design remains fundamental if the challenges of the emerging disease environment are to be met (sensitivity, specificity, use of multiple target genes to avoid drop out and capability to detect unexpected and novel viruses).

A range of new detection technologies has emerged over the last decade. The ones that will make the most impact will be compatible with assay designs that can support a high degree of multiplexing and adaptability to the detection of new viruses. Some of the adaptability will reside in astute primer design using
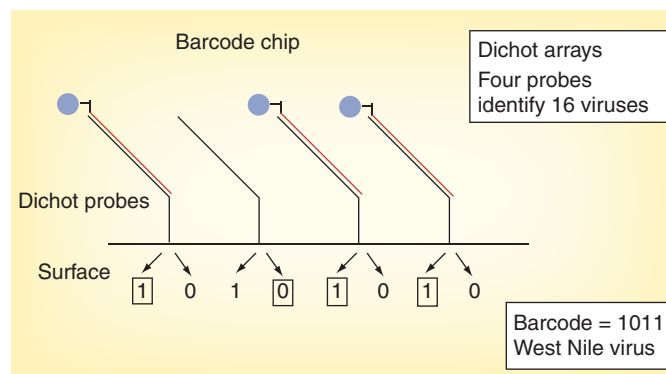

Figure 1. A theoretical barcode that might be generated using a 'dichot' or binary probe array. With the use of only four dichot probes, each generating a yes/no answer, up to 16 viruses can be differentiated. In this theoretical example, three out of four probes bind to the target virus nucleic acid, generating the barcode 1011.
Reproduced with permission from S Maher-Sturgess.

new bioinformatics approaches (see earlier). High degrees of multiplexing, in turn, depend on resolution of signals from simultaneous assays, either by detection at different energy frequencies or by spatially/temporally resolving signals in one, two, three or four dimensions. Azzazy *et al.* reviewed some of the core technologies in nanodiagnostics, comparing quantum dots, cantilevers and nanoparticles [93]. Quantum dots and nanoparticle-based assays offer the potential for large-scale multiplexing of nucleic acid-based diagnostic tests, generally via the use of capture oligonucleotides to address and separate reactions for analysis. Nucleic acid amplification on microspheres is the core technology of new-generation sequencing [94], but is yet to be fully exploited in first-line clinical molecular diagnostics.

At the frontier is research on addressable, real-time nucleic acid tests. These have the potential to be used on microsphere platforms, but need to be interfaced with technology (akin to that used for 454 sequencing [94]) to allow continuous reading of the fluorescent (or other) signal as it develops on microspheres. In one such system under development [95], real-time PCR reactions can be carried out on microspheres (or other substrates, such as microplates or nanowires (see later) using large sets of oligonucleotide sequence tags. With these oligonucleotide tags, one set of microspheres can be adapted to perform many different real-time assays.

Closed, self-contained and fully integrated automated platforms that combine on-board sample preparation with real-time PCR and fluorescence detection are now available, with a range of target-focused, real-time PCR-based diagnostic tests. One assay developed for this type of fully integrated platform is an influenza diagnostic tool that detects and distinguishes (with excellent sensitivity and specificity [96]) between seasonal influenza, influenza B and the novel 2009 H1N1 influenza. However, independent of the efficient hardware used to execute the test, assay design remains the key issue with this type of target-focused assay. Next season, the prevalent strain of influenza A virus may change, necessitating redesign of the assay and renegotiation of the regulatory process.

Although sophisticated assays have been designed to increase the breadth of analytes detected in real-time assays, such as the use of extensions on PCR primers and melt-curve analysis [57], fluorescence systems are dependent on labels and remain limited by the number of optical detection channels. They are therefore limited in the number of target analytes that can be selected, necessitating redesign to accommodate the emergence of novel, recombinant, reassorted or genetic variants.

Nanowires may overcome the challenge of multiplexing because, as no label is required, they are not limited by the number of available labels. Each wire is less than 50 nm wide and may be coated or covalently linked to oligonucleotides. Nanowire arrays, where nucleic acids are amplified and hybridized to nanowires, result in direct conversion of the hybridization event to an electrical signal and direct interface with information systems. Stern and colleagues have developed nanowire complementary metal oxide semiconductor-based label-free immunodetection [97]. To avoid the problem of Debye screening, the salt concentrations in the buffers used for macromolecular sensing experiments must be chosen so that Debye distance is sufficiently long to enable sensing, but sufficiently short to screen unbound macromolecules [97]. Others are developing etched silicon-based nanowire sensors for direct measurement of nucleic acid hybridization [98–100]. Bunimovich *et al.* demonstrated that a single-stranded complementary oligonucleotide is able to significantly change the conductance of a group of 20-nm-diameter silicon nanowires in a 0.165 M solution, by hybridizing to a primary DNA strand that has been electrostatically adsorbed onto an amine-terminated organic monolayer on the nanowires [100].
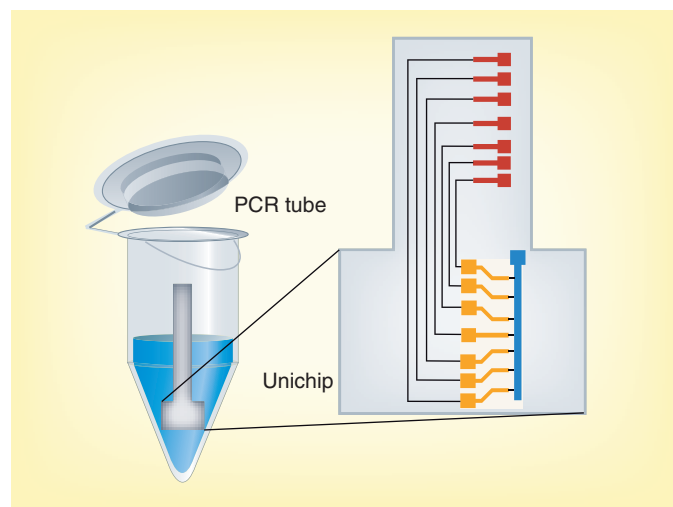

Figure 2. Nanowire chip in PCR tube. Electronic 'drop-in-tube' biochip. The chip could include oligonucleotide-coated nanowires at the lower end of the chip (yellow section), each of which is connected to a discrete radiofrequency identification transponder (red section) [102]. The process by which nucleic acids are coated on the nanowires, and the distance between the wire surface and the hybridization event, will be critical (see [99,100]) to allow this type of device to operate at the ionic strengths encountered in PCR reactions. Reproduced with permission from Biochip Innovations Ltd (Brisbane, Australia).

This intimate contact of the primary strand with the amine groups of the nanowire surface brings the binding event within the Debye distance, allowing the binding event to be electronically detected. Moreover, within a 0.165 M ionic strength solution, the DNA hybridization is efficient, compared with solutions of lower ionic strength [100].

This work will lead to multichannel devices capable of very high degrees of multiplexing. If such systems are interfaced with sets of 'universal' capture oligonucleotides, coated or covalently linked onto the nanowires, they will become highly parallel and rapidly adaptable diagnostic systems. The longer-term vision for these devices is for integration with amplification, using nanowire chips that can be dropped into a PCR tube and relay the electrical signal via radio-frequency identification device from remote locations to a central server (**Figure 2**).

Expert commentary

It is evident that with the rise of techniques such as random PCR, unbiased new-generation sequencing and pathogen detection microarrays, virus diagnosis and virus discovery methods will continue to develop synergistically. Discovery of a new hybridization pattern on a microarray can be translated very quickly into a diagnostic test for a novel virus. Metagenomics has transformed our ability to discover viruses, but is not yet widely used in clinical diagnostic laboratories. However, it has alerted us to the potentially staggering viral diversity that exists and will increasingly inform the design of assays immediately suitable for use in the clinic (e.g., multiplex real-time PCR or microsphere-based virus diagnostic panels). The combination of random amplification PCR with arrays (either broad-spectrum, conventionally designed arrays [71] or simple but powerful arrays, designed using innovative bioinformatics [70]) with new hardware platforms will increase diagnostic capacity and the ability of laboratories to detect unexpected viruses.

The drive to multiplexing is driven by the desire to increase diagnostic capacity and reduce costs. As the technologies and design strategies improve, multiplexed assays can achieve comparable sensitivity to the parent monoplex assays [55,67].

A limitation of real-time PCR using existing technology, is the restriction on multiplexing imposed by the limited number of available fluorophores with separable spectra. However, the number of detectable simultaneous targets has been broadened by intelligent primer design, such as the use of 'tails' on PCR products to allow differentiation by size or melting curve [55] and splitting samples into multiple reactions. A second and pervasive restriction which is a limitation of any target-focused amplification or hybridization nucleic acid test, is the possibility of drop out when the target sequence evolves and novel variants emerge. This continues to plague commercial and in-house assays [35] and necessitates astute primer design or the use of multiple gene targets within a single organism [36,37]. In the emerging disease environment, those within target multiplexing will need to be more generally adopted. Thus, the pressure for increased multiplexing as a result of two requirements: first, the need to increase the number of gene targets within a single virus, and second,

the need to increase the number of different viruses that can be detected in a single assay.

Current front-line virus diagnostic tests take no account of the molecular signatures that carry important information regarding host preference, pathogenicity and drug resistance. This is the case for influenza A virus, for which there is now a good understanding of the molecular basis for host preference (avian versus human), high and low pathogenicity (mutations in *PB2* and *HA* segments) and drug resistance. Incorporating the capacity to detect these signatures is technically feasible and would allow public health responses to be rapidly informed by molecular signatures, in addition to public health statistics on morbidity and mortality, which have proven to be unreliable in some countries where novel influenza A viruses have emerged.

Five-year view

In an emerging disease environment, technologies are required to enable the detection of unanticipated pathogens. The surprise emergences of USUV, WNV and CHIKV in temperate regions were salutory events, as was the rapid emergence of drug-resistant H1N1 influenza A virus in Europe during the 2008–2009 season. Technologies that are rapidly adaptable, impervious to virus evolution and information-rich will be crucial if we are to respond in a timely and informed manner to those surprises, avoiding the need for *de novo* assay design or sequential assays.

The growing trend is towards highly multiplexed assays for infectious agents, using spatially addressable reactions on chips, microplates, microspheres and nanowires. Early examples of this emerging trend are microsphere-based respiratory panels. On the more distant horizon, we may see the emergence of highly multiplexed, real-time PCR assays on microspheres or 2D arrays. This would offer broad-spectrum PCR and multiplex microsphere

detection, combined with quantification. Indeed, such real-time detection technologies are already in development for use on microspheres or other platforms [94].

In the next 5 years, new devices will enter the market, facilitating diagnostic testing at point-of-care, and PCR in the field [101,205]. Pathogen detection arrays will be developed, combining nucleic acid extraction and capture of nucleic acids onto an array with on-board, new-generation sequencing. The integration of these new platforms with appropriate oligonucleotide and primer design (sufficiently flexible to detect emergent and/or variant viruses) will change the face of diagnostics, taking a discovery tool to the clinic and providing more detailed information about the better known pathogens.

As high-throughput, unbiased sequencing becomes cheaper, we will see an increased application of this technique as a diagnostic tool, useful in the absence of *a priori* assumptions based on presumptive diagnosis. For this to become a reality, the medical, veterinary or ecological significance of these newly discovered viruses in disease will need to be elucidated. One can then visualize a discovery-driven increase in research into the biology, pathogenesis and epidemiology of emerging viruses. Evidently, diagnostic virology has a very exciting future.

Financial & competing interests disclosure

Key issues

- The epidemiological environment is changing as a result of interacting biological, climatic and demographic factors.
- This necessitates a change in approach to diagnosis, requiring design of tests that cast a broader net for novel viruses and that lessen the risk of incorrect results by targeting multiple genes in a single virus.
- Technologies that facilitate a broad-spectrum approach to diagnosis will come to the forefront in the next 5 years. These will include highly multiplexed real-time assays, microsphere/nanosphere-based technologies and unbiased high-throughput sequencing.
- It is a reasonable supposition that many novel viruses will be discovered during the next few years through the use of metagenomics techniques including random PCR, new-generation sequencing, arrays and mass spectrometry.
- Metagenomic approaches will gradually be incorporated into routine clinical laboratory workflows; the challenge will be to develop decision criteria for using metagenomics rather than target-focused assays.
- Much research will be necessary to determine whether or not these new viruses are pathogens and to incorporate this knowledge into practical, adaptable diagnostic tests.

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