Review Paper

Rapid diagnosis of viral infections: new tools for monitoring and control of new and emerging diseases

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Introduction

The years at the turn of the millennium have brought home to us a stark reminder that the technological innovations of these last decades have engendered such changes in our way of life and the ecology of our environments that tropical diseases can no longer be thought of as exotic. Indeed the very term exotic belies the perspective from which tropical medicine has been viewed, researched and developed. With the arrival of West Nile virus in New York City in 1999 (CDC, 1999) it has become clear that the recognition of a new disease, hantavirus pulmonary syndrome, in the southwestern United States in 1993 (Nichol, 1993) was not an isolated event for the developed world, and the unusual has become unsurprising. But while we are unsurprised at the unusual we continue to be reminded that Ebola virus is still a danger in Africa, Crimean Congo haemorrhagic fever does still cause death in Pakistan and Japanese encephalitis is a regular cause of encephalitis among children in Sarawak even if it was not the culprit which caused the deaths of more than 100 people in Perak, Selangor and Negeri Sembilan in 1998 and 1999. This initial error in etiological diagnosis (Farrar, 1999) and the subsequent culturing and identification of a new paramyxovirus (Chua *et al.*, 1999) exemplifies the problems associated with the diagnosis, recognition and control of new and emerging diseases. Indeed, with the globalisation of infectious diseases, it has become increasingly necessary to take a syndrome approach to diagnosis in order to avoid the pitfalls of viewing the patient as unconnected to the larger community and to approach etiological identification with a wider vision.

In the past 25 years at least 30 new and re-emerging diseases and syndromes have been recognised (Fauci, 1998). Although the scope of a discussion on new and emerging diseases must include parasites, bacteria and fungi, especially in the light of continually emerging drug resistance and infection of the immunocompromised, this review will be confined to discussing technologies as applied to the etiological diagnosis of viral infections. It is the field of diagnostic virology that the molecular technologies of the past two decades have transformed from a science of retrospective diagnosis to a science of rapid diagnosis, and it is probably partially due to these advances that viral infections are leading the way in being recognised as new and emerging diseases on a global level.

The classical methods

Culturing virus from relevant clinical specimens no doubt provides the most convincing evidence of the presence of a virus, even if the question of causation would still have to be addressed. Virus isolation in various primary cells or continuous cell lines has generated the pathogens for the epidemiological, immunological and molecular characterisation which have formed the basis of modern virology. However, not all viruses are easily cultured, and the study of the diseases associated with such viruses has always been difficult.

Virus isolation and identification of the viruses isolated from clinical specimens is a tedious process requiring skills which can only come from experience. Most diagnostic virology laboratories would have facilities for virus isolation, and such methods have not changed significantly in the last quarter of a century. The arsenal of methods for the identification of viruses cultured however, has vastly increased. The classical tool of virus neutralisation has long been joined by specific identification of virus antigens in cells with monoclonal antibodies using methods such as immunofluorescence or other immunological means of specifically identifying viral antigens.

These immunological tools have, in some cases, been successfully extended to directly identifying viral antigens in clinical specimens without culturing, for example the identification of respiratory syncytial

virus antigens in throat samples using immunofluorescence techniques, or the detection of enteric adenoviruses in stool samples using ELISA.

Not all attempts to culture virus will succeed. Hence it is important to realise that while a positive virus isolation and successful identification of the virus isolated is indisputable evidence of the presence of the particular agent, unsuccessful isolation is not confirmation of absence of the agent. Indeed, virus isolation techniques notoriously lack sensitivity.

Furthermore, even when a virus is easily isolated its identification requires access to specific reagents such as monoclonal antibodies and hyperimmune sera. Most diagnostic laboratories have access only to commercially available reagents and whatever may be prepared in-house. Outbreak monitoring and response requires availability of a panel of antisera which can be applied to the identification of new viruses. Nipah virus was identified as a paramyxovirus related to Hendra virus by such means using antisera at the Centers for Disease Control and Prevention in Atlanta, USA. Comprehensive panels of such reagents are generally exclusively held by a small number of laboratories, but a global initiative for the surveillance and control of new and emerging diseases should require a distribution of these reagents to the regions of the world where these diseases are expected to appear.

Whatever methods are used to identify viruses successfully isolated from clinical material, the limiting factor is the time it takes to grow the isolate. It is because of the slowness and low sensitivity of classical virus isolation techniques that the indirect serological techniques gained dominance in diagnosis of viral diseases. Dissatisfied with negative or non-existent results from virus isolation techniques, doctors can now make assumptions about recent infection by asking questions about the patients' immune response against the virus.

In this regard, the detection of specific immunoglobulins using erythrocyte-based methods such as haemagglutination inhibition and complement fixation required the use of well spaced paired serum specimens to establish seroconversion. While still a retrospective diagnosis, at least an answer was always to be expected. The advent of the enzyme immunoassays such as ELISA allowed detection of specific classes of immunoglobulins and henceforth the detection of IgM in the acute phase immune response to infection became the serological method of choice for acute infections. The sensitivity of the enzyme immunoassays allows demonstration of seroconversion in paired sera taken only a few days apart for both specific IgG and IgM, and yet most doctors still assume the 14 day separation of paired sera is necessary and are slow to take advantage of the leaps in sensitivity which are now the norm in serological diagnosis.

Serological data provides clues to the identity of the infecting virus. However, it is an indirect method and since the human immune response to pathogens will elicit not only antibodies against pathogen-unique antigens but also against antigens and antigenic determinants which are shared by a family of viruses, serological diagnosis may incorrectly identify the agent responsible if there is a lack of awareness of this biological reality. Doctors often call these "non-specific" reactions, but in fact this is inaccurate, because the antibodies are highly specifically directed against antigenic determinants which just happen to be found on more than one member of the family of viruses. This is the problem which lead to the early error in the diagnosis of WNV in New York in the summer of 1999 (Solomon & Cardosa, in press).

Molecular methods

Today it is also possible to identify the viruses isolated using molecular methods such as the polymerase chain reaction (PCR), followed by confirmatory methods such as sequencing or hybridization with specific oligonucleotide probes. There are many reviews of these methods as applied to diagnosis of infectious diseases and the reviews of Louie *et al.* (2000) and Read *et al.* (2000) are examples of useful recent accounts.

PCR uses synthetic oligonucleotide primers applied to different ends of a target DNA sequence to amplify small amounts of DNA template in a reiterative process of denaturation, annealing and extension using heat stable DNA polymerases. This generates a "PCR product" at a geometric rate, making it possible to detect the presence of a particular DNA sequence even if present in very small quantities. Confirmation of the specificity of these reactions can be sought by hybridising oligonucleotide probes specific for internal sequences, and there are several techniques for attaining this goal. RNA templates are reverse transcribed first to obtain cDNA templates which can be used in a subsequent PCR, and this is referred to as RT PCR.

Another less widely applied amplification method is the ligase chain reaction (LCR) which combines the

use of a polymerase with a thermostable ligase to ligate two adjacent oligonucleotides which have annealed to a target template. Like PCR this is also a reiterative process with the original template as well as the newly ligated oligonucleotides providing the templates for the next step, leading to an exponential growth in ligated product, thus rendering detectable small amounts of original template DNA or RNA.

Much has been said about the problems of the amplifications methods, not least being the ability of PCR to pick up minute quantities of contaminating material, hence giving rise to false positive results. As for any laboratory technique, a system of quality assurance must be implemented in order to be able to be immediately cognisant of the possibility of spurious results arising from such problems. The LCR is said to be less plagued with problems of false positives and indeed is expected to be so specific that it can be designed to distinguish between closely related strains of pathogens.

Other criticisms raised against these molecular methods include the issue of cost and the problem of trained human resources. There is no doubt that molecular biology is expensive in terms of cost of materials such as thermostable polymerases and reverse transcriptase. However, any cost-benefit analysis must take into consideration the hidden costs of retrospective or late diagnosis, such as the unnecessary use of antibiotics or antiviral drugs or the public health expenditure in situations where outbreaks occur due to late recognition of a problem.

However, the major failing of the molecular methods routinely used is the requirement of some knowledge of the DNA sequence of the pathogens we want to detect. This requirement thus makes PCR less useful in the monitoring and detection of new or unrecognised pathogens. Surveillance systems which target certain specific pathogens such as avian influenza strains (Webster, 1998) or enterovirus type 71 (Cardosa, unpublished) can use PCR to track the appearance and evolution of the pathogens in question.

The big question is how to recognise new pathogens for which no sequence data is available. One approach to "pathogen discovery" is broad-range PCR (Relman, 1998) where a collection of conserved-region virus family-restricted primer sequences are used to amplify templates which are then sequenced to identify new viruses within a family. This is how a new hantavirus was identified as the agent associated with an acute pulmonary disease in New Mexico, USA in 1993.

A second approach compares fragments of DNA from a matched pair of genetic material and identifies the fragments which are different. Several techniques can be used to obtain this information one of which is representational difference analysis or RDA which was successfully employed to identify HHV8 as a presumptive causative agent of Kaposi's sarcoma (Chang *et al.*, 1994). A third approach uses the immune sera to identify gene products encoded by a new pathogen. This was how hepatitis C virus was identified from an expression library of an infected specimen (Choo *et al.*, 1989).

The future

The time-tested paradigm for laboratory diagnosis of virus infection has been to ask a specific question about the identity of infecting agent: "Is it Japanese encephalitis?" Hence no matter what methods are used to determine yes or no, this paradigm is essentially unable to respond to the needs of rapid response to recognition and control of new diseases. A negative answer leads to the formulation of another specific question, and another wait for the answer. A partial solution to this problem has been sought in what has been referred to as "multiplex PCR" where multiple primer sets are applied to a single specimen so that the test asks more than one question at a time. This solution is however limited by problems of interpretation when applied to primary specimens due to mispriming and does not expand appreciably the range of viruses which can be identified in a single test.

The development of DNA microarrays (Chee *et al.*, 1996; Schena *et al.*, 1996) has changed all this. The technology now exists to spot minute quantities of DNA onto glass slides in organised microscopic arrays (the "DNA chip"). The DNA can be either synthetic oligonucleotides or cloned DNA probes. Target DNA from clinical specimens can be labelled with various fluorescent tags and hybridised to the DNA chip. The binding patterns target sequences of each sample can be read using fluorescence scanning laser devices, and data analysed to determine what probes have been bound. This technology facilitates simultaneous detection of several thousands of genes. Arising from the advances of the human genome project, DNA microarrays were first applied to the study of differential gene expression in non-communicable diseases, but in 1999 Chambers and

co-workers described the first DNA microarray of a virus, the cytomegalovirus genome.

So far, DNA microarrays have contained probes for many genes of large genomes and research questions have been directed at differences in gene expression patterns in various disease processes or host-pathogen responses. It is surely possible to harness this powerful technology to produce DNA chips which contain probes for as many human pathogens as possible which might be etiologically responsible for a particular syndrome. In this way the detection of the presence of any of these pathogens may be conducted simultaneously and if the DNA microarray contains strain-specific as well as family-generic probes it would also be possible to identify new microbes to family level. The selection and testing of each of these probes would be a major task and would require close collaboration between different expert groups and needs careful coordination, but this revolution in the diagnosis of infectious diseases will make the whole process more efficient, more objective and more amenable to the new paradigm of telemedicine and telehealth.

Obviously these approaches to the discovery of new pathogens will require simultaneously a discourse on the question of causation, a debate already eloquently started by the questions raised by Fredricks & Relman (1996) about rethinking Koch's postulates.

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